THE CLINICAL SIGNIFICANCE OF LOSS OF DNA MISMATCH REPAIR IN OVARIAN CANCER PATIENTS; AN IMMUNOHISTOCHEMICAL STUDY

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DECLARATION

I, Melanie Mackean, declare that all the results presented in this thesis are my own work and that I have composed this thesis in its' entirety. I have not presented this thesis for any other degree, diploma or professional qualification.

Date.....13-11-02.....

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ABBREVIATIONS USED

% - Percentage Score

Ab – Antibody

ABC – Avidin Biotin Peroxidase Complex

ADP – Adenosine diphosphate

ATP – Adenosine triphosphate

BOC - Beatson Oncology Centre

Cisplatin – *Cis*-dichlorodiammineplatinum

DAB – Diaminobenzidine

DM - Dr Millan scores

DNA – Deoxyribose Nucleic Acid

EGFR – Epidermal Growth Factor Receptor

EIA – Enzyme Immuno Assay

EORTC – European Organization for Research and Treatment of Cancer

ER – Oestrogen Receptor

FIGO – International Federation of Gynecologic Oncologists

GSE – Genetic Suppressor Elements

GSH – Glutathione

HMG – High Mobility Group Proteins

HNPCC – Hereditary Nonpolyposis Colon Cancer

HR – Hazard Ratio

H-score – Histoscore

I – Intensity Score

IC50 – Concentration of drug inducing a 50% reduction in the surviving fraction

IC90 - Concentration of drug inducing a 90% reduction in the surviving fraction

IHC - Immunohistochemistry

IRS – Immunoreactive Score

LOH – Loss of Heterozygosity

MJM - Dr Mackean scores

MLH1 – Mut L homologue 1

MMR – Mismatch DNA repair

MNNG – N-methyl-N'-Nitro-N-nitrosoguanidine

MNU – N'-methyl-N-Nitrosourea

MSH2 – Mut S homologue 2

MSI – Microsatellite instability

NER – Nucleotide Excision Repair

PCNA – Proliferationg Cell Nuclear Antigen

PgR – Progesterone Receptor

RD – Residual Disease

RER – Replication Error Phenotype

SLE – Systemic Lupus Erythematosus

TTP – Time to Progression

WHO – World Health Organisation

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Chapter 1 - ABSTRACT

Loss of mismatch repair (MMR) and, in particular, loss of MLH1 is associated with acquired cisplatin resistance of ovarian tumour cell line models. The aim of this thesis is to examine in ovarian cancer patients the clinical prognostic significance of the MMR proteins MLH1 and MSH2 when measured at disease presentation. We have developed immunohistochemistry (IHC) and a scoring system for the expression of MLH1 and MSH2 in paraffin embedded tissues. We have scored both the intensity of staining (I-score) and percentage of cells staining (%-score). We have validated this technique with good agreement in scoring over time, between blocks and between observers (inter-observer kappa scores of ≥ 0.629, intra-observer kappa scores of ≥ 0.646 and intra-slide kappa scores of ≥ 0.583). There was a positive correlation of Ki67, (a proliferation marker), with I-MLH1, %-MLH1 and %-MSH2 scores ($p=0.002$; <0.001 and 0.001) but not with p53 scores.

We examined prechemotherapy samples from 58 patients with a histological diagnosis of advanced ovarian carcinoma, who were then treated with primary chemotherapy regimens containing cisplatin. Advanced stage was associated with increased percentage cells positive for MLH1, MSH2 and Ki67 ($p = 0.0092$, 0.0049 and 0.0054 respectively). We performed a multivariate analysis allowing for known clinical prognostic factors, (i.e. type of chemotherapy given, stage, performance status and residual disease). Patients with a loss of intensity of staining for MMR proteins prechemotherapy showed a poor survival (Hazard Ratio, HR=3.64; p=0.0012) and poor progression free survival (HR=2.37; p=0.016). Similarly patients showing a reduced intensity of MLH1 staining showed a poor overall survival (HR=2.17; p=0.031). There was no correlation of MMR proteins and tumour response to treatment.

Loss of mismatch repair (MMR) in ovarian tumour cells after cisplatin-based chemotherapy has been shown in both *in vitro* and *in vivo*. We have examined paired samples pre and post chemotherapy in 26 ovarian cancer patients. We have seen no consistent changes in MMR proteins, p53 or Ki67 pre and post chemotherapy. On multivariate analysis change in MMR expression did not correlate with survival but it was found that a reduction in intensity of MSH2 staining post chemotherapy was associated with a longer progression free survival (HR 0.52; p=0.011).

Although further prospective validation studies will be necessary, our observations support the proposal that low MLH1 and/or MSH2 expression is associated with resistance *in vivo* of ovarian tumours to cisplatin and hence poor survival of patients after cisplatin-based chemotherapy.

Chapter 2 – AIMS OF STUDY

I. To develop reliable immunohistochemistry for mismatch repair proteins in archival, paraffin embedded, ovarian tumour samples from patients.

   A. Technique development

   B. Interobserver variation

   C. Intraobserver variation

   D. Range of expression of proteins

   E. Correlation with p53 and Ki67 expression

   F. Expression in differentiated tissue (endometrial model)

II. To correlate expression of mismatch repair proteins by immunohistochemistry to the following:

   A. Response to cisplatin based chemotherapy

   B. Progression free survival

   C. Overall survival

   D. Clinical prognostic factors

III. To examine sequential ovarian tumour samples taken from the same patient before and after chemotherapy for expression of mismatch repair proteins by immunohistochemistry.

   A. Overall differences

   B. Correlation with clinical outcome

Chapter 3 – INTRODUCTION

3 (i) DNA MISMATCH REPAIR

A – GENERAL INTRODUCTION

A (I) DNA repair in humans (eukaryotes)

DNA repair systems act to maintain genomic integrity in the face of replication errors, environmental insults, and the cumulative effects of age. DNA repair can be defined in a general sense as a range of cellular responses associated with the restoration of the genetic instructions provided by the normal primary DNA sequence. More than 70 human genes directly involved in the five major pathways of DNA repair have been described (Yu, Chen et al. 1999). The five pathways are not mutually exclusive but are described briefly below.

1. **Direct Repair**. The simplest response to DNA damage is to remove or reverse the lesion in a single step reaction, restoring the local sequence to its original state. Human enzymes involved include 6-4 photolyase and O^6-methyl-guanine DNA methyltransferase (MGMT). MGMT is known to be important in repair of alkylation damage.

2. **Base Excision Repair (BER).** This targets 'nonbulky' base adducts such as those produced by methylation, oxidation, reduction, or fragmentation of bases by ionizing radiation or oxidative damage. The removal of the modified base by a DNA glycosylase is the major feature of BER. A pair of apurinic endonucleases then incises the 3' and 5' next to the apurinic site. DNA polymerase β fills in the gap and DNA ligase seals the nick. There are 5 characterised human DNA

glycosylases with different but occasionally overlapping substrate specificity. DNA ligases are a common pathway to BER and NER.

3. **Nucleotide Excision Repair (NER).** NER repairs bulky DNA adducts including thymine dimers and 6-4 photoproducts. The major enzyme involved, excinuclease, is a complex of at least 16 polypepetides. NER involves four stages; damage recognition, incision, gap-filling and ligation.

4. **Double strand break repair**. Double strand breaks (DSB) arise in DNA under physiological conditions e.g. somatic recombination, but are also produced by ionizing radiation and oxidative insults. Mammalian cells primarily rejoin DSBs by nonhomologous mechanisms but homologous repair is also found. Homologous DSB repair involves physical interactions of RAD51, RAD52 and RPA. Nonhomologous end-joining pathways of DSB repair begin with binding of the free ends by DNA dependent protein kinase (DNA-PK). After binding of subunits XRCC7, XRCC5 and XRCC6, DNA-PK is activated for its phosphorylation activity involving XRCC4 and DNA ligase IV.

5. **Mismatch Repair (MMR).** Mismatched base pairs arise through processes including misincorporations during DNA replication, formation of heteroduplexes and secondary structures such as imperfect palindromes. Mismatches can also be the results of deamination of 5-methylcytosine to thymidine resulting in a G:T mismatch. In MMR a patch of nucleotides is removed from one strand, followed by repair synthesis and ligation. This type of repair is the focus of this thesis and shall now be discussed in more detail.

A (II) DNA mismatch repair (MMR)

The most extensively characterised general mismatch repair system is the *Escherichia coli* MutHLS system, which repairs a broad spectrum of mispaired bases and has been reconstituted with purified enzymes. Eukaryotes are known to contain a MMR system that contains at least some components that are highly related to key components of the bacterial system, shown in table 1.

Table 1 – Components of the human DNA mismatch repair system

HUMAN ENZYME	SIZE AND GENE LOCATION	*E. coli* HOMOLOG	*S. cerevisiae* HOMOLOG	FUNCTION
hPMS1	106kDa 2q31-33	Mut-L	PMS1	Unknown.
hPMS2	96kDa 7p22	Mut-L	PMS1	Forms heterodimer with hMLH1.
hMLH1	85kDa 3p22-23	Mut-L	MLH1	Forms heterodimer with hPMS2.
hMSH2	105kDa 2p22-21	Mut-S	MSH2	Interacts with either hMSH3 or hMSH6. Recognition of mismatch, ATPase.
hMSH3	127kDa 5q11-q13	Mut-S	MSH3	Forms a heterodimer with hMSH2.
hMSH6	153kDa 2p16	Mut-S	MSH6	Forms a heterodimer with hMSH2.

The standard hypothesis of the mechanism of action of MMR presents a 'looping model' in which MSH proteins form a complex with MLH proteins to bind

mismatched nucleotides, thus creating a machine that translocates bi-directionally from the mismatch to an 'activation'site. In *E.coli* this would be the MutH protein bound to transiently hemi-methylated GATC sites in newly replicated DNA (Kolodner 1996). Activation of MutH results in single strand scission and loading of the excision repair machinery, which leads to replacement of the error-containing DNA strand. This model is confounded by the lack of MutH homologs or a methylation requirement for MMR in humans.

A different model has been suggested by Fischel's group in which MSH proteins function as an adenosine nucleotide regulated molecular switch (Fischel, Acharya et al. 1999). In this 'sliding clamp' model MSH proteins survey DNA in the ADP-bound form. When DNA mismatch is detected, it provokes ADP to ATP exchange by the MSH subunits which causes a conformational change, leading to the formation of a hydrolysis-independent sliding clamp in which MSH proteins encircle the DNA. In the ATP-activated form, the sliding clamp is thought to tranduce a 'mismatch signal' to a downstream effector such as the polymerase machinery, which controls excision repair. In the absence of excision repair or when presented with an overwhelming amount of DNA damage, the ATP-activated form of hMSH2-hMSH6 signals directly to the apoptotic machinery. The bacterial MutL homologs, may serve as the ATPase accelerating protein, which induces the hydrolysis of ATP and release of the hMSH2-hMSH6 heterodimer from the DNA, which recycles the signalling switch. They suggest that DNA damage may signal the apoptotic machinery directly through ATP-activated MSH proteins thus bypassing p53. Loss of MMR would therefore prevent the damage-induced apoptotic response enabling for improved survival of neoplastic cells, or DNA damaged cells.

A (III) MMR and Hereditary Non Polyposis Colon Cancer (HNPCC)

Loss of MMR causes destabilization of the genome and results in high mutation rates, particularly in microsatellite sequences in both noncoding and coding portions of the genome. This so called 'microsatellite instability' (MSI) has been used as a marker for loss of MMR and is also called the 'replication error prone' phenotype (RER+). Such microsatellite sequences are found in the coding regions of the HPRT, APRT, APC, type II TGF-*B* receptor and BAX genes (Fink, Aebi et al. 1998). Mutation rates are increased at these loci in MMR deficient cells. The majority of HNPCC cancer cases are due to mutations in either MLH1 or MSH2 (Liu, Parsons et al. 1996). Although the MMR system seems to be normal in the heterozygote germ line cells containing a single functional gene copy, during carcinogenesis, the remaining wild-type allele is somatically mutated which results in the complete loss of MMR function in the tumour. In addition to HNPCC, loss of MMR occurs frequently in many types of sporadic cancers including endometrial, small and non-small cell lung, pancreatic, gastric, ovarian, cervix and breast cancers (Han, Yanagisawa et al. 1993; Risinger, Berchuck et al. 1993; Merlo, Mabry et al. 1994; Shridhar, Siegfried et al. 1994; Wooster, Cleton-Jansen et al. 1994).

B – DNA MISMATCH REPAIR AND OVARIAN CANCER

MSI and ovarian cancer

Microsatellites are widely distributed, repetitive DNA sequences composed of short, tandemly repeated nucleotide motifs. In some neoplasms these sequences exhibit a form of genetic instability characterised by the gain or loss of repeat units at multiple independent loci. These lead to a change in size of an allele in the tumour compared to the germline allele. This is called microsatellite instability (MSI) and such tumours are reported as showing the replication error (RER) positive phenotype. The 6 published reports on MSI in sporadic ovarian cancer are shown in table 2.

MSI for all the studies was defined as a change in size of an allele in any one of the markers examined. The overall incidence of MSI was found to vary between 0% and 17% of sporadic ovarian tumours. The interpretation of these varying observations is partially confounded by the small size of the studies and the variables of family history, tumour histology, tumour stage and type of microsatellite marker studies.

Two studies found an increased incidence of MSI in endometrioid histology (5 out of 10 and 2 out of 5 samples; i.e.50% and 40% respectively) compared to other epithelial ovarian tumour subtypes (Fujita, Enomoto et al. 1995) (King, Carcangiu et al. 1995). King et al. also noted an increased incidence in non-epithelial subtypes (40%) but was the only study to examine this in any detail (King, Carcangiu et al. 1995). The only other study to quote the histology of cases showed no MSI in the two cases of endometrioid tumour examined (Osborne and Leech 1994). If there is a higher

incidence of MSI in endometrioid and non-epithelial ovarian tumours the lack of MSI found in the study by Dodson et al. may in part be accounted for by histological case mix (i.e. if the majority of tumours were serous) (Dodson, Thibodeau et al. 1993).

Table 2 – Frequency and associations of MSI in sporadic ovarian cancer

STUDY	MSI (%)	PRIMERS USED	ASSOCIATIONS	COMMENTS
(Dodson, Thibodeau et al. 1993)	0/60 (0%)	At least 20 markers used (?dinucleotide repeats)	N/A	N/A
(Han, Yanagisawa et al. 1993)	3/19 (15.8%)	4 dinucleotide repeats	None examined	Histology and stage not quoted.
(Osborne and Leech 1994)	2/25 (8%)	Nucleotide repeats not used	None examined	Histology and stage quoted.
(Wooster, Cleton-Jansen et al. 1994)	2/20 (10%)	3 tetra-, 3 tri-, 6 dinucleotide repeats	None examined	Histology and stage not quoted. MSI in tri- and tetranucleotide repeats but not dinucleotides.
(Fujita, Enomoto et al. 1995)	8/47 (17%)	4 dinucleotide repeats	More common in advanced stage endometrioid cancers	
(King, Carcangiu et al. 1995)	7/41 (17%)	1 tetra-, and 1-dinucleotide repeats	Increased in endometrioid and non-epithelial types. Increased in stage I. Not related to family history	7 with MSI at tetranucleotide repeat. Only 2 at dinucleotide repeat.

Ovarian tumours occur with relatively high frequency in some HNPCC families (Lynch, Bewtra et al. 1986). It is often difficult to obtain a sufficient family history to exclude the possibility that the cases are indeed sporadic and not familial. The only study to examine family history in detail found no increase in MSI in the 12 cases with a positive family history compared to the 27 cases without an affected family member (King, Carcangiu et al. 1995). In this limited study they did not find family history identified any HNPCC families, suggesting that the majority of familial ovarian cancers are not related to HNPCC.

In colorectal cancer there is evidence that tumours exhibiting MSI tend to present at an earlier stage (Thibodeau, Bren et al. 1993). In ovarian cancer the association of MSI and stage has only been examined in 2 of the above studies. King et al. showed a significant association of MSI with stage I tumours ($p=0.001$) (King, Carcangiu et al. 1995). They found tumour associated alterations in 3 out of 4 (75%) of stage I tumours versus 4 out of 37 (11%) of the stage II, III, and IV patients. Conversely Fujita et al. found no overall association with stage and MSI (Fujita, Enomoto et al. 1995). On subgroup analysis they found a significant trend for MSI to be associated with advanced stage in the 10 tumours of endometrioid type studied ($p=0.025$), but the numbers were small.

A consensus has been reached in the classification of MSI in colon cancer as to the number and type of nucleotide repeats to be used for definition of MSI (Boland, Thibodeau et al. 1998). No such work has been done in ovarian cancer and the consensus statement warns against extrapolation of such guidelines to other tumour

types. In the above studies different types and numbers of primers have been used to examine for MSI. In one study the microsatellite markers were chosen for allelotyping of ovarian cancer and dinucleotide repeats were not specifically used (Osborne and Leech 1994). This may account for the low figure of 8% of MSI found. Likewise the study that found no MSI in 60 cases, commented on the use of CA dinucleotide repeats, but the markers which were used are not fully shown in this study which has only been reported in abstract form (Dodson, Thibodeau et al. 1993). In contrast 2 studies have shown an increased yield of MSI using tri- and tetranucleotide repeats compared to dinucleotide repeats (Wooster, Cleton-Jansen et al. 1994; King, Carcangiu et al. 1995) in ovarian cancer. In vitro observations suggest that small mismatches and large loops resulting from slippage events at repeat sequences may be recognised and repaired by different components of the DNA mismatch repair machinery (Umar, Boyer et al. 1994). Small mismatches involving only a few bases would be more likely to arise in dinucleotide repeats, whereas large loops are more likely to result form slippage at tri- and tetranucleotide repeats. Although dinucleotide instability has been seen in some studies (Han, Yanagisawa et al. 1993; Fujita, Enomoto et al. 1995) the increased instability with tri- and tetranucleotide repeats suggest that some sporadic ovarian neoplasms may be specifically defective for large loop DNA repair activity.

None of the above studies have specifically examined the relationship of MSI and prognosis in ovarian cancer. In particular the response to chemotherapy has not been examined.

Conclusions

Microsatellite instability in sporadic ovarian tumours:

- Occurs in around 10-15% of cases.
- May be more common in ovarian tumours of endometrioid and non-epithelial histological subtypes.
- May be more common in stage I tumours.
- Is more commonly found with tri- and tetra-nucleotide than dinucleotide repeats.
- Has not been examined with regard to prognosis.

C – LOSS OF DNA MISMATCH REPAIR AND ASSOCIATION WITH CHEMOTHERAPY RESISTANCE

Evidence is accruing that loss of one the mismatch repair genes can also lead to resistance to certain types of chemotherapy, including cisplatin, in mammalian cells. The first piece of evidence was from a study in *Escherichia coli* into methylator resistance (Fram, Cusick et al. 1985). They showed loss of sensitivity to cisplatin by loss of MMR in methylase deficient cells. There are now several lines of evidence linking loss of MMR to cisplatin chemotherapy resistance.

C (I) In vitro evidence for loss of MMR in cisplatin resistant cell lines.

- *Acquisition of the microsatellite instability (RER+) phenotype in chemotherapy resistant cell lines.*

Anthoney et al. showed acquisition of the microsatellite instability (RER+) phenotype in 9 out of 10 ovarian cancer cells lines with *in vitro* acquired cisplatin resistance (Anthoney, McIlwrath et al. 1996). They confirmed this microsatellite instability was seen in a stable cisplatin resistant cell line but not the cisplatin sensitive parental cell line suggesting this was a stable loss of MMR function and not induced temporarily by chemotherapy. Likewise, a human ovarian adenocarcinoma cell line 2008 showed no microsatellite instability whereas the cisplatin resistant derivative 2008A readily showed microsatellite instability (Aebi, Kurdi-Haidar et al. 1996).

- *Loss of expression of MMR genes in chemotherapy resistant cell lines.*

Subsequent work in independent, cisplatin resistant derivatives of a human ovarian cancer cell line showed that acquisition of the microsatellite instability (RER+)

phenotype was due to loss of expression of the hMLH1 and hPMS2 protein subunits of the MutLα MMR complex which occurred as a frequent event (9/10). This was shown to be due to decreased expression of only hMLH1 mRNA and not hPMS2 mRNA (Brown, Hirst et al. 1997). Similarly the cisplatin resistant ovarian cancer cell line 2008A showed loss of expression of hMLH1 protein on Western immunoblot compared with the parental 2008 cell line (Aebi, Kurdi-Haidar et al. 1996).

Similar work on cisplatin resistant melanoma cell lines (MeWo CIS 0.01 and MeWo CIS 1) compared to the parental cell line (MeWo) showed a 0.3 fold reduction in hMSH2; a 0.6 fold reduction in hMLH1 and a 0.7 fold reduction in hMSH6 protein expression by Western blot analysis respectively. On Northern blot analysis of mRNA only the MeWoCIS1 cell line showed a reduction in mRNA of 0.2 fold for hMSH2 and 0.6 fold for hMLH1 compared to the parental cell line. Interestingly neither of the cisplatin resistant cell lines showed microsatellite instability suggesting that a reduction in MLH1 or MSH2 expression may occur that is sufficient to be associated with cisplatin resistance but not the RER+ phenotype (Lage, Christmann et al. 1999).

- *Loss of MMR function in chemotherapy resistant cell lines.*

Deficiency in strand specific mismatch repair has been shown in two cisplatin resistant ovarian cell lines compared to the parental cell line (Drummond, Anthoney et al. 1996). The defect in repair of either a single G-T mispair or a -CA- dinucleleotide insertion/deletion mismatch was evident in the resistant cell lines only when the strand break that directs the reaction was located 3' to the mismatch. DNA repair was restored by the addition of purified MutLα heterodimer but not the MutSα heterodimer (Drummond, Anthoney et al. 1996).

Similarly a cisplatin resistant derivative (A2780/CP70) ovarian cancer cell line showed a reduction in a G:T mismatch recognition activity, by gel retardation assay, compared to the parental cell line (Anthoney, McIlwrath et al. 1996).

Loss of MMR has been shown to markedly increase the mutation rate at the *HPRT* locus. The cisplatin resistant, hMLH1 deficient, ovarian cancer cell line 2008A showed a mutation rate at the *HPRT* locus that was elevated between 10.8 to 139-fold compared to the cisplatin sensitive, hMLH1 present, parental 2008 cell line. This suggests a mutator phenotype exists due to the loss of DNA mismatch repair function (Aebi, Kurdi-Haidar et al. 1996).

- *Restoration of MMR genes leading to restoration of chemotherapy sensitivity.*

Because it has proved difficult to transfect cell lines with MMR genes alone, much of this work has been done using transfection of complete chromosome 3 (containing hMLH1) and chromosome 2 (containing hMSH2). HCT116 is a human colorectal adenocarcinoma cell line deficient in hMLH1. It has been shown that HCT116 +ch2 (hMLH1 deficient) was 2 fold resistant to cisplatin compared to a subline complemented with chromosome 3 expressing a wild-type copy of hMLH1 (Fink, Nebel et al. 1996). Likewise, the human endometrial cancer cell line HEC59, which is deficient in hMSH2, was 1.8 fold resistant to cisplatin in clonogenic assays when compared to a subline complemented with chromosome 2 containing wild-type hMSH2 (Aebi, Kurdi-Haidar et al. 1996). This 2 fold resistance is of the order to be of clinical significance.

Using isogenic strains of *Saccharomyces cerevisiae* it has been shown that disruption of the MutS homologues MSH2, MSH3 and MSH6 and the MutL homologues MLH1 and MLH2 conferred a significant increase in resistance to cisplatin compared with

the wild type. Transformation of the MLH1 gene back into the MLH1 mutant strain led to increased sensitivity to cisplatin compared with vector-alone control (Durant, Morris et al. 1999).

- *The MMR complex hMutSα can recognise and bind to the cisplatin 1,2 crosslink.*

Using binding assays with a series of duplex oligonucleotides containing a single 1,2 diguanyl intrastrand crosslink typical of cisplatin damage (Yamada, O'Regan et al. 1997) showed that human cell extracts contain factors that preferentially recognise this type of damage. Interestingly this binding was only recognised when the complementary strand contains T opposite the 3' and C opposite the 5' guanine in the crosslink. In cell lines deficient in hMutSα (LoVo and DLD-1) the binding activity was absent. Confirming this finding purified human hMutSα exhibits the same substrate preference for platinated DNA. This data suggests that it is the hMutSα complex that binds to the 1,2 diguanyl intrastrand crosslink (Yamada, O'Regan et al. 1997).

It has also been shown by mobility shift assays that there is formation of protein-DNA complexes that contain hMSH2 and hMLH1 when nuclear extracts are incubated with DNA platinated with cisplatin (Fink, Nebel et al. 1996).

- *Loss of MMR function leads to inability to undergo chemotherapy-induced apoptosis.*

Independent cisplatin resistant derivatives of a human ovarian cancer cell line also lose the ability to undergo cisplatin induced apoptosis, which is associated with loss of p53 function (Anthoney, McIlwrath et al. 1996; Drummond, Anthoney et al. 1996). Other cellular responses to cisplatin induced damage have been shown to be

abrogated by loss of MMR. The cisplatin resistant, hMLH1 deficient, ovarian cancer cell line A2780/CP70 loses the ability to engage G1 and G2 cell cycle arrest after cisplatin damage compared to the hMLH1 intact parental cell line (Brown, Hirst et al. 1997). Restoration of hMLH1 also leads to restoration of ability to engage G2 arrest (Buermeyer, Van Patten et al. 1999)

- *Enrichment for MMR deficient cells occurs during treatment with cisplatin.*

It has been shown that treatment with cisplatin can increase the percentage of cells with loss of MMR. Using HCT116 (a colon tumour cell line deficient in hMLH1) sublines complemented with chromosome 2 (control hMLH1 deficient) versus chromosome 3 (hMHL1 proficient) in a 95:5 ratio Fink et al. showed a 53% enrichment, 5 days after a one hour exposure to IC50 concentration of cisplatin, for MMR deficient cells. This enrichment increased with multiple exposures to cisplatin by up to 77% after the fourth exposure. Enrichment was more pronounced when the cells were exposed to an IC90 concentration of cisplatin and after the fourth cycle there were 163% more MMR deficient cells (Fink, Zheng et al. 1997).

Using a MMR deficient (clone B) and MMR proficient (clone B/rev) pair of Chinese hamster ovary sublines Fink et al. showed a 1.8 fold resistance to cisplatin by clonogenic assays for clone B cells. Five days after a single 1-hour exposure to cisplatin at an IC50 concentration, populations containing 5 or 10% clone B cells showed enrichment for clone B cells of 81 and 75% respectively. This enrichment was more pronounced with increased drug exposure. Following an IC90 concentration exposure to cisplatin the increases were 158 and 169% respectively (Fink, Nebel et al. 1998).

C (II) In vivo evidence for loss of MMR in cisplatin resistance

- *In vivo resistance to cisplatin in MMR deficient tumours in mice models.*

In this study MSH2-/- and MSH2+/+ embryonic stem cells were established as xenografts in athymic nude mice. Each mouse had 2 tumours of each type. 48 hours after implantation the animals were treated with a single LD10 dose of cisplatin. The remaining mean volumes of the MSH2-/- tumours were statistically significantly larger than those of the MSH2+/+ tumours ($p<0.0001$). This suggested that loss of MSH2 alone resulted in a resistance to treatment with cisplatin (Fink, Zheng et al. 1997).

- *Increase (enrichment) in cells with loss of MMR in cisplatin exposed tumours.*

A 5:95% ratio of Chinese hamster ovary sublines of MMR deficient (Clone B) and MMR proficient (clone B/rev) were grown as xenografts in mice. A single LD10 dose of cisplatin enriched the tumours from 4.6% to 6.8% MMR-deficient cells. This represented an enrichment of 48% (just significant at $p=0.04$) for MMR deficient cells (Fink, Nebel et al. 1998).

An increase in the proportion of ovarian tumours negative for the hMLH1 subunit has been observed in samples taken at second look laporotomy after cisplatin/cyclophosphamide chemotherapy (36%; 4/11) compared to untreated primary tumours (10%; 4/39) (Brown, Hirst et al. 1997). No difference is seen in expression of hMSH2, hMSH6 or hPMS2 by immunoblot Western analysis. Unfortunately, in this retrospective study these samples were not paired i.e. they did not come from the same patients pre and post chemotherapy.

In a separate study paired ovarian cancer tumour samples were obtained prior to and after 3 cycles of either cisplatin or carboplatin based chemotherapy in 38 patients. The expression of hMLH1 was examined using immunohistochemistry. The number of tumour cells showing positive staining for MLH1 was scored, in decades, blindly by an independent pathologist. 66% of the paired samples showed a reduction in hMLH1 expression after the chemotherapy (p=0.0005) (Fink, Nebel et al. 1998).

In summary, there is a growing body of evidence linking loss of mismatch repair with resistance to cisplatin chemotherapy, but only 2 studies suggest that this is relevant in treatment of tumours in man (Brown, Hirst et al. 1997; Fink, Nebel et al. 1998).

C (III) Loss of mismatch repair and resistance to other chemotherapy agents.

There are numerous studies linking the loss of mismatch repair with resistance to other chemotherapy agents. The initial work was on the methylating agents MNNG, MNU and the purine analogue, 6-thioguanine, and has been confirmed in newer agents such as temozlamide. However, following the results that MMR loss was also important in cisplatin resistance, several other drugs have been shown to have altered chemosensitivity dependent upon MMR status. These include the cisplatin alternative carboplatin, but interestingly not the derivative oxaliplatin. Also this includes doxorubicin, and the topo II isomerase inhibitor etoposide. Recently the clinical treatment of ovarian cancer has come to include taxoids as first line and topo I isomerase inhibitors as 2^{nd} line chemotherapy but there is no evidence that MMR status alters sensitivity to paclitaxel or topotecan. Table 3 lists the evidence for each of the cytotoxic drugs followed by a summary of the studies included.

Table 3 - Summary of evidence for drug resistance associated with loss of MMR

DRUG	Acquisition of RER+ phenotype associated with resistance	Loss of MMR gene expression associated with resistance	Loss of MMR function associated with increased resistance	Restoration of MMR genes confers sensitivity	Other evidence
MNNG	Y (13)	Y (13)	Y (13)	Y (11, 18)	
MNU	Y (9, 6)	Y (15, 6)		Y (8, 9)	
Carboplatin		Y (7)		N (7) Y (11, 14, 16)	Enrichment for MMR loss (16)
Doxorubicin	Y (12)	Y (7, 12)	Y (12)	Y (7, 16) N (11)	Clinical evidence (10). Enrichment for MMR loss (16)
Etoposide	N (3)	Y (3)		Y (11, 16)	Enrichment for MMR loss (16)

Vindesine	N (3)	Y (3)			
Fotemustine	N (3)	Y (3)			
Temozolamide	Y (6)	Y (6)			Negative on clinical evidence (5)
6-Thioguanine	N (13)	Y (15) N (13)	N (13)	Y (2, 11, 16)	6-TG induced cell cycle arrest restored by MLH1 (2). Enrichment for MMR loss (16)
DNA minor groove alkylating agents i.e. tallimustine, carzelelsin, CC1065		Y (1)		Y (1)	
DNA non-covalent minor groove binder PNU 151807		N (1)		N (1)	
Ultra violet XR		N (7)		N (7)	
Paclitaxel				N (11, 16)	No enrichment for MMR loss (16)

5FU (5-Fluorouracil)		Y (19)		N (11) Y (19)	
Perfosfamide				N (11, 16)	No enrichment for MMR loss (16)
Cyclophosphamide	N (6)	N (6)			
Melphalan				N (16)	No enrichment for MMR loss (16)
Tamoxifen				N (16)	No enrichment for MMR loss (16)
Topo I isomerase inhibitors, (Topotecan, 9-aminocamptothecan)	N (6)	N (6)			
Oxaliplatin		N (4)		N (4, 14)	No change in DNA replicative bypass (4). No enrichment for MMR loss or difference in *in vivo* response (17)

MNNG = N-methyl-N'-Nitro-N-nitrosoguanidine; MNU = N'-methyl-N-Nitrosourea

- *Summary of studies examining chemotherapy resistance and loss of MMR*

1. (Colella, Marchinin et al. 1999) Used HCT116, a human colorectal adenocarcinoma cell line, (hMLH1 deficient) versus HCT116+chromosome 3 (hMLH1 proficient). Also used A2780 (MMR proficient) versus A2780/CP70 and A2780/MCP-1 (both hMLH1 deficient) versus A2780/CP70 +chromosome 3 (hMLH1 proficient). Showed loss of MMR was associated with resistance to three alkylating minor groove binders (Tallimustine, Carzelesin and CC1065) but not to non-covalent minor groove binder PNU151807. Restoration of MMR by chromosome transfer led to restoration of chemosensitivity to the alkylating minor groove binders.

2. (Buermeyer, Van Patten et al. 1999) Used a mouse embryonic fibroblast cell line (MEF) deficient in hMLH1 and transfected with cDNA for *hMLH1*. Showed resistance to 6-thioguanine in wild type MEF compared to hMLH1 deficient MEF. Showed some restoration of chemosensitivity to 6-TG by transfection with hMLH1. Also showed that restoration of hMLH1 restored 6-TG induced cell cycle arrest.

3. (Lage, Christmann et al. 1999) Used MeWo, a human melanoma cell line, and selected subclones for chemotherapy resistance. Showed loss of MMR proteins hMLH1, hMSH2 and hMSH6 in cells resistant to etoposide and vindesine, but not fotemustine. Showed reduction in mRNA encoding hMLH1 and hMSH2 in cells resistant to etoposide and fotemustine but only a small reduction in cells resistant to vindesine. Interestingly did not show RER+ve phenotype in resistant cell lines despite reductions in MMR protein expression.

4. (Vaisman, Varchenko et al. 1998) Used several models of MMR proficient versus MMR deficient cells lines including: A2780 versus A2780/CP70 (hMLH1 deficient); HCT116 (hMLH1 deficient) versus HCT116 +ch3 versus HCT 116 +ch2; HHUA (hMSH3 and hMSH6 deficient) versus HHUA+ch5 (hMSH3 proficient) versus HHUA+ch2 (hMSH6 proficient) and DLD-1 (hMSH6 deficient) versus DLD-1+ch2 (hMSH6 proficient). Showed loss of MMR proteins by Western blot in each cell line but no change in sensitivity to oxaliplatin in MMR proficient versus MMR deficient systems. Also showed no change in DNA replicative bypass of oxaliplatin adducts in association with loss of MMR.

5. (Freidman, McLendon et al. 1998) Showed no significant difference in response rate in 38 newly diagnosed malignant glioma patients treated with temozolamide by immunohistochemistry for MMR proteins hMLH1 and hMSH2.

6. (Friedman, Johnson et al. 1997) Used D-245MG, a human glioblastoma multiforme xenograft compared to D-245MG(PR) subline. Showed loss of hMSH2 protein and microsatellite instability in the (PR) subline compared to the parental subline. The MMR deficient subline was resistant to procarbazine, temozolamide, MNU, and busulfan but not to BCNU, cyclophosphamide, topotecan, CPT-11, and 9-aminocamptothecin.

7. (Durant, Morris et al. 1999) Used a yeast model with genetic knockouts of MMR genes. Showed loss of MSH2, MSH3, MSH6, MLH1 and MLH2 but not PMS1 increased resistance to carboplatin 1.5 to 3.1 fold. Likewise loss of MSH2, MSH3, MSH6 and MLH1 increased resistance to doxorubicin 2.1 to 6 fold. Restoration of the MLH1 gene into MLH1 knockouts restored sensitivity to doxorubicin but not significantly to carboplatin.

8. (Moreland, Illand et al. 1999) Used the A2780/CP70 MLH1 deficient cell line complemented by either chromosome 2 (control) or chromosome 3 (MLH1 +). The MMR defective cell line was more resistant to MNU than the MMR proficient cells.

9. (Umar, Koi et al. 1997) Used an endometrial tumour cell line HEC 59 (deficient in hMSH2) and colon tumour cell line HCT15 (deficient in hMSH6) compared with complemented cell lines containing chromosome 2 (MSH2 and MSH6 proficient). Showed that restoration of MMR restored sensitivity to MNU and also Microsatellite stability.

10. (Mackay, Cameron et al. 2000) Primary chemotherapy with either epirubicin or doxorubicin containing regimens in 29 breast cancer patients resulted in a significant increase in the percentage of tumour cells with reduced hMLH1 immunohistochemical staining ($p=0.01$). This enrichment for loss of hMLH1 was strongly associated with poor disease free survival ($p=0.0025$) suggesting chemotherapy resistance in vivo.

11. (Aebi, Fink et al. 1997) Used HCT116 (a colon tumour cell line deficient in hMLH1) and compared sublines complemented with chromosome 2 (control hMLH1 deficient) or chromosome 3 (hMHL1 proficient). Also used HEC59 (a human endometrial adenocarcinoma cell line) deficient in hMSH2 versus a complemented cell line with chromosome 2 (hMSH2 proficient). Showed increased resistance to MNNG, 6-thioguanine, cisplatin, carboplatin and etoposide with loss of hMLH1, and loss of hMSH2 but not to perfosfamide, melphalan, 5-Flurouracil, doxorubicin or paclitaxel.

12. (Drummond, Anthoney et al. 1996) Showed loss of MLH1 protein and reduction of hPMS2 protein in nuclear extracts of doxorubicin resistant subline (A2780/AD) compared to parental doxorubicin sensitive cell line (A2780). Also showed defect in strand specific mismatch repair in doxorubicin resistant cell line that was restored by addition of purified MutLα heterodimer.

13. (Aebi, Kurdi-Haidar et al. 1996) Used 2008, a human ovarian adenocarcinoma cell line and 2008/A, a subline selected for cisplatin resistance. Showed resistant subline was RER+ve compared to parental subline. Also showed loss of hMLH1 protein in resistant subline. Confirmed functional loss of MMR by HPRT mutation assay. The mutation rate was elevated 65 fold in the resistant compared to the sensitive cell line. They showed the cell line 2008/A (hMLH1 deficient) was also resistant to MNNG but surprisingly was 2.6 hypersensitive to 6-thioguanine compared to the parental cell line (hMLH1 proficient).

14. (Fink, Nebel et al. 1996) Used HCT116 (a colon tumour cell line deficient in hMLH1) and compared sublines complemented with chromosome 2 (control hMLH1 deficient) versus chromosome 3 (hMHL1 proficient). Also used HEC59 (a human endometrial adenocarcinoma cell line) deficient in hMSH2 versus a complemented cell line with chromosome 2 (hMSH2 proficient). Showed a 1.3 fold and 1.5 fold resistance to carboplatin in the MMR deficient cell line compared to complemented MMR proficient cell lines HCT116 and HEC59 respectively. Showed no difference in MMR proficient and deficient cell lines in sensitivity to oxaliplatin (also tetraplatin, transplatin, JM335 or JM216).

15. (Brown, Hirst et al. 1997) Used A2780, a human ovarian adenocarcinoma cell line and 10 cisplatin resistant sublines and one doxorubicin resistant cell line A,

which were shown to be deficient in hMLH1 and hPMS2 protein but only hMLH1 mRNA. Showed cross-resistance to MNU and 6-thioguanine in MMR deficient cell lines compared to MMR proficient parental cell line.

16. (Fink, Nebel et al. 1998) Used HCT116 (a colon tumour cell line deficient in hMLH1) and compared sublines complemented with chromosome 2 (control hMLH1 deficient) or chromosome 3 (hMHL1 proficient). Showed resistance in MMR deficient cell line for carboplatin, etoposide, 6-thioguanine and (in contrast to their earlier study) doxorubicin. Also showed enrichment for loss of MMR cells after single treatment with these drugs. No change in chemosensitivity or enrichment seen with melphalan, paclitaxel, perfosfamide or tamoxifen.

17. (Fink, Zheng et al. 1997) Used HCT116 (a colon tumour cell line deficient in hMLH1) and compared sublines complemented with chromosome 2 (control hMLH1 deficient) versus chromosome 3 (hMHL1 proficient). Showed lack of enrichment for MMR deficient cells after treatment with oxaliplatin. Also showed in nude mice transplanted with isogenic embryonic tumour cells which were both MSH2 deficient and proficient that there was no difference according to MMR status and response *in vivo* to oxaliplatin.

18. (Koi, Umar et al. 1994) Used HCT116 (a colon tumour cell line deficient in hMLH1) and compared sublines complemented with chromosome 2 (control hMLH1 deficient) versus chromosome 3 (hMHL1 proficient). Showed a 200 fold reduced tolerance to MNNG by restoring MMR activity with chromosome 3.

19. (Carethers, Chauhan et al. 1999) Used HCT116 (a colon tumour cell line deficient in hMLH1) and compared sublines complemented with chromosome 2 (control hMLH1 deficient) versus chromosome 3 (hMHL1 proficient). Also used SW480

(MMR proficient), LoVo and 2774 (hMSH2deficient) cell lines. Showed 28 fold resistance in MMR deficient cell lines to 5FU compared to MMR proficient cell lines.

D – DNA MISMATCH REPAIR AND IMMUNOHISTOCHEMISTRY

D (I) Previous work on MMR immunohistochemistry technique

In 1995 Wilson et al. first described immunohistochemistry for MSH2 only in paraffin embedded biopsies of the small and large intestine (Wilson, Ewel et al. 1995). They used a rabbit polyclonal antibody to MSH2 (Oncogene) with autoclave heat antigen retrieval at 65°C for one hour plus an overnight incubation. The colon and ileum showed strong staining in crypts of Lieberkuhn. Proliferative perifibroblasts in colon also showed significant presence of MSH2. They concluded that MSH2 is expressed in highly proliferative cells of the gut. They also examined expression of RNA for MSH2 in various normal human tissues including skin, lung, skeletal muscle, heart, liver, spleen, thymus, prostate, testis, ovary, small intestine, colon and leukocytes by Northern blot analysis and showed a high expression in testis and thymus.

Leach et al. followed with confirmatory study (Leach, Polyak et al. 1996). They developed a mouse monoclonal Ab to hMSH2 (FE11) carboxyl terminal to use on frozen tissue with an overnight incubation. They noted that MSH2 expression was exclusively nuclear and expressed in a variety of human tissues including thyroid, heart, smooth muscle and the germinal centres of lymphoid follicles. There was prominent staining for MSH2 in the proliferative compartment of the epithelium of

the digestive tract, extending from oesophagus to rectum. This was shown to be similar to staining for Ki67. Using material from 2 patients with HNPCC, they showed lack of staining in the adenomas and carcinomas but normal staining of the surrounding stromal cells. Immunohistochemistry for PMS2 has proved a little more problematic. The only report of PMS2 immunohistochemistry is by Fink et al. examining the expression of hMLH1 and hPMS2 in frozen sections of normal human tissues (Fink, Nebel et al. 1997). They used overnight incubation with MLH1 (mouse monoclonal; PharMingen) and PMS2 (rabbit polyclonal; Santa Cruz) antibodies. Again they saw exclusively nuclear staining with very prominent staining in the proliferating epithelia of the digestive tract. They were the first to show strong staining in primitive testicular germ cells. Interestingly in the ovary they showed staining of the nuclei of the granulosa cells and a subset of the stromal cells but the surface epithelium, from which the majority of ovarian carcinomas are thought to derive, and the germ cells were surprisingly non-reactive.

Conclusions

- Immunohistochemistry is feasible for MLH1 and MSH2 in paraffin embedded material with heat antigen retrieval.

- Immunohistochemistry is feasible for PMS2 in fresh frozen material but has not been shown for paraffin embedded material.

- MMR staining appears to be localised to the proliferative compartment in normal human gut tissues.

- MMR staining is also seen in the proliferative compartment of normal human thymus and testis, but not in the epithelial component of normal human ovary.

D (II) MMR immunohistochemistry compared to microsatellite analysis

An important study by Thibodeau et al. compared MMR immunohistochemistry to MSI and gene mutation (Thibodeau, French et al. 1996). Using heat induced antigen retrieval they examined MSH2 (Oncogene) and MLH1 (Pharmingen) expression in 32 paraffin embedded colorectal cancer biopsies. 4 patients did not have enough material for MSI analysis. The results are shown in table 4 below.

Table 4 – Comparison of MSI and MMR immunohistochemistry.

Thibodeau et al	Immunohistochemistry absent (loss of MMR*)	Immunohistochemistry present (normal)	Totals
MSI present (loss of MMR*)	14 True positive	5 False negative	19
No MSI present (normal)	0 False positive	9 True negative	9
Totals	14	14	28

*Detection of **loss of MMR** is the definition of a 'positive' test for immunohistochemistry.

They used strict criteria of complete absence of staining of tumour cells in presence of positively staining non-neoplastic cells to be scored 'negative' for immunohistochemistry. All 14 tumours with loss of immunohistochemical staining were found to have MSI i.e. immunohistochemistry had a low false positive rate and high specificity. All 9 of the MSI negative tumours had normal

immunohistochemistry staining. However, 5 out of 14 tumours with normal immunohistochemistry had MSI i.e. were falsely negative. Interestingly only one out of these 5 tumours had a mutation to account for the presence of MSI.

They also compared MMR gene mutation and immunohistochemistry and the results are shown in table 5. Overall only 8 of the 19 MSI positive tumours had gene mutations. Immunohistochemistry identified 7 out of these 8 with loss of staining. Of the 7 cases* with loss of immunohistochemistry that were MSI positive but mutation negative, 6 of these involved loss of MLH1 immunohistochemistry. One could speculate that these mutation negative MSI tumours might be due to epigenetic factors such as hypermethylation of the promoter to cause loss of gene function.

Table 5 - Comparison of MSI, MMR immunohistochemistry and MMR gene mutation.

Thibodeau et al	MSI present, immuno lost	No MSI, immuno lost	MSI present, immuno normal	No MSI, immuno normal	Totals
MMR gene mutation present	7	0	1	0	8
No gene mutation	7*	0	4	9	20
Totals	14	0	5	9	28

Bocker et al. showed that loss of immunohistochemistry depends on the degree of MSI (Bocker, Palazzo et al. 1999). They compared staining for MLH1 and MSH2 in 45 colorectal cancer patients, either under age 50 or with a family history of HNPCC, to microsatellite instability. They showed loss of immunohistochemical staining is much more likely to be associated with high microsatellite instability, shown in table 6.

Table 6 - Comparison of MSI and MMR immunohistochemistry.

Bocker et al	loss of immuno	normal immuno	Totals
MSI-High	14 (93%)	1	15
MSI-Low	1 (13%)	7	8
MSI-Stable (normal)	2 (9%)	20	22
	$p<0.00001$		45

Parc et al. examined the expression of MLH1 and MSH2 in paraffin embedded material from 62 endometrial carcinomas in young patients (age < 52) (Parc, Halling et al. 1999). They confirmed the low false positive rate for immunohistochemistry, shown in table 7. All the patients with no microsatellite instability had normal immuno-histochemistry. However almost 25% of MSI positive tumours had not lost immunohistochemistry but they did not examine for reductions in intensity or the percentage of cells stained.

Table 7 - Comparison of MSI and MMR immunohistochemistry.

Parc et al	loss of immuno	normal immuno	Totals
MSI +ve	16	5	21
MSI-ve	0	41	41
	16	46	62

Hartmann et al. confirmed that loss of immunohistochemistry is very specific for MSI +ve tumours of the ureter and renal pelvis i.e. all MSI negative tumours show normal immunohistochemistry for MLH1 and MSH2, shown in table 8 (Hartmann, Zanardo et al. 1999).

Table 8 - Comparison of MSI and MMR immunohistochemistry.

Hartmann et al	loss of immuno	normal immuno	Totals
renal pelvis MSI +ve	3	0	3
renal pelvis MSI -ve	0	34	34
ureter MSI +ve	10	2	12
ureter MSI-ve	0	19	19
	13	55	68

Recently Marcus et al. have shown that immunohistochemistry for hMLH1 and hMSH2 is highly sensitive and specific for prediction of MSI in colorectal tumours,

shown in table 9 (Marcus, Madlensky et al. 1999). They also tested 16 cases with known germline mutations in either hMLH1 or hMSH2 and showed that immunohistochemistry for the correct MMR protein was lost in all 16 cases (100% sensitivity).

Table 9 - Comparison of MSI and MMR immunohistochemistry.

Marcus et al	loss of immuno	normal immuno	Totals
MSI +ve	37	1	38
MSI-ve	0	34	34
	37	35	72

There has only been one large study examining immunohistochemistry for MMR as a surrogate test for defective mismatch repair compared to MSI (Cawkwell, Gray et al. 1999). All MSI positive tumours were identified by the immunohistochemical test for loss of hMLH1 or hMSH2, using heat activated antigen retrieval. They examined 215 MSI negative cases and found all to have positive staining for hMLH1 and hMSH2, shown in table 10. They did not compare immunohistochemistry with gene mutation.

Table 10 - Comparison of MSI and MMR immunohistochemistry.

Cawkwell, L	loss of immuno	normal immuno	Totals
MSI +ve	82	0	82
MSI-ve	0	215	215
	82	215	297

Combining the above studies, shown in table 11, allows us to calculate the specificity, sensitivity and power of positive test of MMR immunohistochemistry compared to microsatellite analysis in detection of loss of MMR, shown in table 12.

Table 11 – Combined results on all studies of MSI versus MMR immunohistochemistry

	MSI present, immuno lost	No MSI, immuno lost	MSI present, immuno normal	No MSI, immuno normal	Totals
	True positive	False positive	False negative	True negative	
Thibodeau et al	14	0	5	9	28
Bocker et al	15	2	8	20	45
Parc et al	16	0	5	41	62
Hartmann et al	13	0	2	53	68
Marcus et al	37	0	1	34	72
Cawkwell et al	82	0	0	215	297
Totals	177	2	21	372	572

Table 12 – Sensitivity and Specificity of MMR immunohistochemistry to predict MSI

Sensitivity and specificity of immunohistochemistry compared to gold standard of MSI.		**'Test'** Immunohisotchemistry		Totals
		+ve lost	-ve present	
'True' MSI	+ve MSI present	177	21	198
	-ve No MSI	2	372	374
Totals		179	393	572

Sensitivity = $\frac{\text{True +ve who are test +ve}}{\text{All true +ve}} = \frac{177}{198} = 89\%$

Specificity = $\frac{\text{True –ve who are test –ve}}{\text{All true -ve}} = \frac{372}{374} = 99.5\%$

Predictive power of a +ve test (i.e. loss of immunohistochemistry) =

$$\frac{\text{Sensitivity x proportion of true +ve}}{(\text{Sensitivity x proportion of true +ve}) + (\text{1-specificity x proportion of true –ve})} =$$

$$\frac{0.89 \times (198/572)}{(0.89 \times (198/572)) + ((1-0.995) \times (374/572))} = 98.9\%$$

Combining the two limited studies that examined gene mutation and loss of immunohistochemistry gives the following results, shown in table 13.

Table 13 – Combined results of MMR immunohistochemistry versus MMR gene mutation

	Gene mutation, immuno lost	No gene mutation, immuno lost	Gene mutation, immuno normal	No gene mutation, immuno normal	Totals
	True positive	False positive	False negative	True negative	
Thibodeau et al	7	7	1	15	30
Marcus et al	16		0		16
Totals	23	7	1	15	46

Using these results allows us to estimate the predictive power of MMR immunohistochemistry compared to MMR gene mutation, shown in table 14.

Table 14 – Sensitivity and Specificity of MMR immunohistochemistry to predict MMR gene mutation

Sensitivity and specificity of immunohistochemistry compared to gene mutation		**'Test'** Immunohisotchemistry		Totals
		+ve lost	-ve present	
'True' Gene mutation	+ve Gene mutation present	23	1	24
	-ve No gene mutation present	7	15	22
Totals		30	16	46

Sensitivity = $\frac{\text{True +ve who are test +ve}}{\text{All true +ve}} = \frac{23}{24} = 95.8\%$

Specificity = $\frac{\text{True –ve who are test –ve}}{\text{All true -ve}} = \frac{15}{22} = 68\%$

Predictive power of a +ve test (i.e. loss of immunohistochemistry) =

$$\frac{\text{Sensitivity x proportion of true +ve}}{\text{(Sensitivity x proportion of true +ve) + (1-specificity x proportion of true –ve)}} =$$

$$\frac{0.958 \times (24/46)}{(0.958 \times (24/46)) + ((1-0.68) \times (22/46))} = 76.5\%$$

Conclusions

- Total loss of MMR immunohistochemistry tends only to occur in MSI positive tumours.

- Not all MSI positive tumours show complete loss of immunohistochemical staining.

- Nearly all MSI negative tumours show normal immunohistochemical staining.

- The sensitivity of immunohistochemistry to detect loss of MMR compared to MSI analysis is 89%.

- The specificity immunohistochemistry to detect loss of MMR compared to MSI analysis is 99.5%.

- The predictive power of a loss of immunohistochemistry to detect loss of MMR compared to MSI analysis is 98.9%.

- In a limited number of patients immunohistochemistry appears to be very sensitive (96%) compared to gene mutation analysis for loss of MMR but not very specific (68%). Therefore the predictive power of a loss of immunohistochemistry to detect loss of MMR compared to loss of the MMR gene by mutation analysis is only 77%.

3(ii) - OVARIAN CANCER

A (I) – General introduction

Over 90% of malignant tumours of the ovary arise from the surface epithelium, and are the subject of this thesis. The remainder of ovarian tumours arise from the stroma and germ cells and are not further discussed. In Scotland ovarian cancer is the fourth commonest cause of cancer related deaths (age standardized mortality of 20 per 100,000 population) and one in 70 women will develop ovarian cancer in their lifetime (Swerdlow, Silva et al. 1998). There has been a gradual decrease in mortality in younger age groups from ovarian cancer probably reflecting the introduction of effective chemotherapy in the 1970s. In the cohort aged 35-44 years the 5 year survival has improved from 35% for cases occuring in 1968-72 to 56% for cases from 1983-1987 (Swerdlow, Silva et al. 1998). However, epithelial ovarian cancer is predominantly a disease of older women with a median age at diagnosis of 63 years. The highly lethal nature of this tumour is related to the absence of symptoms in the majority of women with early stages of the disease and unfortunately 70% of women present with advanced disease.

In women presenting with symptoms of ovarian cancer an exploratory laparotomy is often necessary for diagnosis. The pathological distinction between epithelial ovarian cancer and other tumours is usually straightforward. The common histological types of epithelial ovarian cancer are detailed in the table 15 below. However, there is often heterogeneity within any given tumour. Some areas may be predominantly serous with other areas predominantly mucinous.

Not only can there be difficulties in heterogeneity of histology there is often difficulty in obtaining material for studies such as the current study due to the fact that surgery may be undertaken in a wide variety of hospitals with different pathology departments. The current study required liaison with 12 different pathology departments throughout the West of Scotland. This can lead to variation in reporting of histological subtypes. In this study all the blocks were subject to central review as part of the original studies and the histology is taken from these.

Table 15 – Histological typing of epithelial ovarian cancer

HISTOLOGICAL TYPES OF MALIGNANT EPITHELIAL OVARIAN TUMOURS (Krigman, Bentley et al. 1994)	CLINICAL CORRELATES
Serous (Papillary)	The commonest type
Endometrioid	Bilateral in 10% of cases
Mucinous	Often with normal CA125 levels
Clear cell	Stage for stage, the worst prognosis
Brenner	Transitional cells, resembles bladder
Mixed epithelial	
Undifferentiated	
Unclassified and miscellaneous	

Once epithelial ovarian cancer has been confirmed, a total abdominal hysterectomy, bilateral salpingo-oophorectomy, and omentectomy are usually performed, accompanied by a careful examination of all serosal surfaces, biopsies of grossly involved areas and collection of ascites or peritoneal washings for cytological studies. If the disease appears to be limited to the ovary, the retroperitoneal nodes are also examined. An attempt is made to debulk all gross disease, and the amount of residual peritoneal or serosal implants remaining at the completion of surgery is noted (Allen, Bak et al. 1993). The staging system defined by the International Federation of Gynecologic Oncologists (FIGO) shown in table 16 (FIGO 1987) assumes that an adequate staging operation, as above, has been performed.

'Advanced epithelial ovarian cancer' is taken to be stage IC or above for the remainder of this thesis. Stage IA and IB and borderline tumours, treated by surgery alone, will not be dealt with further.

Table 16 – Staging system for epithelial ovarian cancer

FIGO staging system for epithelial ovarian cancer **(FIGO 1987)**	
Stage I	**Tumour limited to the ovaries**
IA	One ovary, no ascites, intact capsule
IB	Both ovaries, no ascites, intact capsule
IC	Ruptured capsule, capsular involvement, positive peritoneal washings or malignant ascites
Stage II	**Ovarian tumour with pelvic extension**
IIA	Pelvic extension to uterus or tubes
IIB	Pelvic extension to other pelvic organs (bladder, rectum, vagina)
IIC	Pelvic extension, plus findings as for IC
Stage III	**Tumour outside the pelvis or with positive lymph nodes**
IIIA	Microscopic seeding outside the true pelvis
IIIB	Gross deposits <=2cm
IIIC	Gross deposits >2cm, or positive lymph nodes
Stage IV	**Distant organ involvement**, including liver parenchyma and positive pleural cytology

A (II) - Primary (1st line) chemotherapy

Patients who have residual disease or are at high risk of recurrence (usually stage IC or above) are offered combination chemotherapy that includes a platinum analogue, either cisplatin or carboplatin as standard treatment. Up until recently this would be in combination with cyclophosphamide without doxorubicin (Anon 1994). In the light of 2 recent large randomised trials showing an improvement in overall survival in advanced ovarian cancer for cisplatin-paclitaxel combination compared to cisplatin-cyclophosphamide, the current recommendation is for a cisplatin-paclitaxel combination as first line chemotherapy (Piccart, Du Bois et al. 2000).

B - CLINICAL PROGNOSTIC FACTORS IN EPITHELIAL OVARIAN CANCER

There are a large number of studies attempting to identify subgroups of patients with good or poor prognosis in ovarian cancer using clinical factors. In one of the largest meta-analysis of over 3,000 ovarian cancer patients in 38 trials the following were found to be the main prognostic factors, in order of importance, predicting an improved survival in advanced ovarian epithelial cancer (Voest, van Houwelingen et al. 1989):-

1. Chemotherapy including cisplatin as initial treatment.
2. Residual disease of <2cm post operatively.
3. Good WHO performance status (0,1).
4. FIGO stage II/III compared to stage IV.

On multivariate analysis the type of chemotherapy and amount of residual disease were found to be the most important factors predicting survival. In other studies examining clinical prognostic factors histology, tumour grade, presence or absence of ascites have also been found to be important (Malkasian, Melton et al. 1984; Neijt, ten Bokkel Huinink et al. 1991; Kosary 1994; Makar, Baekelandt et al. 1995). Interestingly referral to a specialist multidisciplinary (MTD) team and a specialist gynaecologist with an interest in oncology has also been shown to improve survival (Junor, Hole et al. 1994). The most consistent factors emerging from nearly all studies of this type are the performance status, the size of residual tumour prior to

chemotherapy and the FIGO stage (Neijt, ten Bokkel Huinink et al. 1991; Brinkhuis, Meijer et al. 1995).

In view of the above, any study looking for molecular markers predictive of outcome in ovarian cancer must try to allow for the clinical prognostic factors that are already known.

Conclusions for collection of samples for prognostic study:

1. All patients should be treated by a MTD team, under a similar protocol.
2. All patients should receive the same chemotherapy or standard chemotherapy as part of a protocol.
3. Full staging should have been performed by a competent gynaecologist and FIGO stage and residual disease noted.
4. Performance status should be noted.
5. If possible, histology, grade of tumour and presence of ascites noted. In particular tumours of borderline malignancy should be excluded.

Ultimately 75% of all patients either do not respond or relapse following cisplatin based chemotherapy and other predictors of response or survival are needed (Neijt, ten Bokkel Huinink et al. 1991).

C - MOLECULAR PROGNOSTIC FACTORS IN OVARIAN CANCER

C (I) - Ovary cancer and p53 status

Somatic mutations of the p53 gene have been described in 30-70% of ovarian cancers. An intact p53 pathway is important in cell cycle control. P53 is dramatically up regulated after DNA damage and leads to cell cycle arrest at the G1-S checkpoint through activation of p21. Arrest of cell proliferation allows for the time necessary for the cell machinery to repair DNA damage before the cells commit themselves to mitosis, thus ensuring preservation of genomic stability. The p53 gene also plays an important role in the activation of apoptosis. P53-dependent apoptosis is mediated by pathways independent of p21 involving up-regulation of pro-apoptotic genes such as *bax* and *bcl-*x_s, as well as down-regulation of survival genes such as *bcl-2*, *bcl-*x_l and *mcl-1* (Coukos and Rubin 1998).

- ***In vitro evidence for p53 and ovarian cancer chemotherapy resistance.***

Analysis of p53 status in human ovarian epithelial cell line has provided evidence that this suppressor gene may be involved in cisplatin resistance. The experimental development of cisplatin resistance in vitro in IGROV-1 cells (an ovarian cancer cell line) is accompanied by the development of p53 mutation and abrogation of the normal response to DNA damage, including up regulation of p53 and related downstream genes such as p21 and *bax* (Perego, Giarola et al. 1996). The ovarian cancer cell line A2780 carries the wild-type p53 gene (Vasey, Jones et al. 1996),

whereas A2780-CP20 subclones displaying 20-fold increased resistance to cisplatin were found to harbour p53 gene mutations (Skilling, Squartrito et al. 1996). Transfection of the A2780 ovarian cancer cell line with a temperature sensitive mutant p53 induced resistance to cisplatin; inhibited chemotherapy induced apoptosis; and was accompanied by a delay in drug-mediated arrest in S-phase (Eliopoulos, Kerr et al. 1995). Similarly transfection of A2780 with a dominant negative mutant p53 was followed by the development of cross-resistance to cisplatin, doxorubicin and ionizing radiation but not paclitaxel (Vasey, Jones et al. 1996).

- ***P53 immunohistochemistry***

The mutant p53 gene can be a dominant negative, i.e. even in the heterozygous state a mutant p53 gene can lead to loss of p53 function as a result of inhibition of the wild-type protein by the mutant p53 protein. P53 normally exists as a tetramer, but participation of mutant protein forms leads to both loss of function as well as to stabilisation of the molecule, resulting in a paradoxical nuclear overexpression of the protein. This nuclear overexpression can be detected by positive immunohistochemical staining.

However, not all p53 alterations lead to overexpression. Overexpression of p53 protein correlates significantly with allelic loss and missense mutations in exons 5-9 (Kohler, Marks et al. 1993) but nonsense mutations, splicing mutation, and most deletions do not result in p53 protein accumulation (Wynford-Thomas 1992; Coukos and Rubin 1998). The latter type of p53 mutations will lead to false negative

immunohistochemistry results. In addition some studies show that overexpression of p53 protein may occur without gene mutation (Kohler, Marks et al. 1993; Kappes, Milde-Langosch et al. 1995) leading to false positive immunohistochemistry results. Concordance of results between gene mutation detection and immunohistochemistry (i.e. both positive or both negative) was 69% in one study (Dix, Robbins et al. 1994). Also different immunohistochemistry techniques e.g. different p53 antibodies, antigen retrieval techniques and fixation, will also affect p53 positivity (Lambkin, Mothersill et al. 1994; Baas, van den Berg et al. 1996). Therefore clinical prognostic studies that rely on immunohistochemical detection of overexpression of p53 as a marker of loss of p53 function may be of limited significance. In an editorial on this subject Hall and Lane conclude that 'p53 immunohistochemistry may indeed prove to be of real value in tumour pathology – but only if pathologists take appropriated care in recording and interpreting the results' (Hall and Lane 1994).

- ***Previous immunohistochemical studies with p53 and clinical outcome in ovarian cancer***

There have been 17 studies which have examined the relationship between p53 immunohistochemistry and clinical outcome in ovarian cancer patients (Marks, Davidoff et al. 1991) (Kohler, Kerns et al. 1993) (Diebold, Baretton et al. 1996) (Bosari, Viale et al. 1993) (Renninson, Baker et al. 1994) (Hartmann, Podratz et al. 1994) (Henriksen, Srang et al. 1994) (Sheridan, Silcocks et al. 1994) (van der Zee, Hollema et al. 1995) (Klemi, Pylkkanen et al. 1995) (Righetti, Torre et al. 1996) (Goff, Muntz et al. 1998) (Silvestrini, Daidone et al. 1998) (Herod, Eliopoulos et al.

1996) (Geisler, Geisler et al. 1997) (Rohlke, Milde-Langosch et al. 1997) (Marx, Meden et al. 1998).

The results from these studies have been confusing and contradictory. On examination for a relationship with survival only 4 out of 9 studies were shown to have a significant association on multivariate analysis for overexpression of p53 associated with poor survival (Klemi, Pylkkanen et al. 1995; Herod, Eliopoulos et al. 1996; Geisler, Geisler et al. 1997; Rohlke, Milde-Langosch et al. 1997). On univariate analysis for survival 9 out of the 13 studies that investigated an association of survival with p53 found a positive association of p53 overexpression with poor survival. Of the 5 studies that investigated response to chemotherapy only one found p53 overexpression was associated with a poor response to chemotherapy (Righetti, Torre et al. 1996) and 4 found no relationship.

Some of the positive studies for p53 immunohistochemistry as a predictor for survival on univariate analysis can be explained by the consistent finding of p53 staining being associated with other known clinical prognostic factors. Of the 14 studies that assessed the relationship of stage with p53 staining, 6 found an association with positive staining and advancing stage, a known poor prognostic factor. Similarly of the 14 studies that examined grade, 7 found that high grade of tumour (i.e. poorly differentiated) was associated with p53 positive staining. Interestingly, 5 out of 12 studies showed a positive correlation of p53 staining with serous histology. Only 8 studies evaluated the relationship of residual disease following laparotomy with p53 staining and 3 found an association and 5 did not. No studies examined the

relationship of p53 staining with performance status. Some studies have been limited in their analyses due to sample size but 7 studies had over 100 patients with one study having 284 patients (Hartmann, Podratz et al. 1994).

Technical details in the immunostaining varied considerably between studies. Different primary antibodies, slide fixation, antigen retrieval techniques and incubation techniques result in an inability to directly compare the various studies. With respect to the scoring of slides only one study gave a measure of reproducibility of scoring between 2 observers (van der Zee, Hollema et al. 1995). Many studies used only one scorer and in only 2 studies were the scorers stated as being blinded to clinical outcome (Kohler, Kerns et al. 1993; van der Zee, Hollema et al. 1995). Only one study used image analysis (Geisler, Geisler et al. 1997) for scoring as opposed to a visual technique. Different cut-off points were used for description of 'positive' staining for p53, although the commonest approach was to classify any degree of positive staining as positive.

Of note, there has been one important study looking not at p53 immunohistochemistry but at gene mutations and their relationship to clinicopathological features and outcome in ovarian cancer (Niwa, Itoh et al. 1994). In 53 patients there was an increase in p53 gene mutations in serous and endometrioid histology ($p<0.05$) but no association with age, stage, or grade of tumour. No association was found with response to chemotherapy or overall survival and p53 gene mutation. Importantly there have been no prospective studies examining the role of p53 immunohistochemistry as a prognostic indicator in ovarian cancer.

C (II) - Ovary cancer and proliferation index as measured by Ki67

Proliferation is one of the most fundamental of biological processes because of its role in growth and in the maintenance of tissue homeostasis. In tumours in particular, proliferation has traditionally received much attention. Ki67 is a proliferating cell nuclear antigen coded by a gene located on chromosome 10. It is correlated with the mitotic cycles and cell growth. Ki67 increases its own expression in the last period of S phase and more in G1, G2 and mitosis, but is not detectable in G0 i.e. it is absent in resting or non-proliferating cells (Brown and Gatter 1990). The percentage of cells expressing Ki67 is therefore a good surrogate marker for the proliferation index. The Ki67 score partly correlates with other proliferation markers like %S-phase cells and mitotic count (van Diest, Brugal et al. 1998).

There were 3 main reasons for examining Ki67 immunostaining in this study;

1. Proliferation, as measured by Ki67 antigen expression, has been shown to be an independent predictor of survival in epithelial ovarian cancer in several studies detailed below.

2. hMSH2 has been shown to localise in cells of the proliferating compartment in normal tissues on immunohistochemistry (Wilson, Ewel et al. 1995; Leach, Polyak et al. 1996).

3. It has been shown that both hMLH1 and hMSH2 can bind to PCNA (proliferating cell nuclear antigen) suggesting a link between MMR and replication (Umar, Buermeyer et al. 1996).

We therefore thought it was important to show that any pathological clinical correlation of MMR proteins in ovarian cancer was independent of a relationship of MMR protein expression with proliferation.

- ***The evidence for an association of Ki67, as a marker of proliferation, with clinical pathological features and outcome in ovarian cancer.***

The initial studies using Ki67 as a proliferation marker in ovarian cancer had to be done on fresh frozen samples using the Dako Ki67 antibody (Jordan, Kerns et al. 1993) and therefore numbers of samples were limited. Despite this they showed a correlation on univariate analysis of increased Ki67 expression with poor overall survival ($p<0.01$). Because of the small numbers, clinical and pathological features and therefore a multivariate analysis of survival could not be performed.

Since the development of the MIB-1 antibody, which can stain for Ki67 antigen in paraffin embedded, historical samples, using a microwave antigen retrieval technique, larger and more detailed studies have been performed. MIB-1 asessments in paraffin sections are more reproducible than their counterparts in frozen sections (van Diest, Brugal et al. 1998). The scoring system used for the 'proliferation index' has varied between studies. Some studies have used a manual percentage positive score with or without the help of a grid (Altavilla, Marchetti et al. 1996; Rohlke, Milde-Langosch et al. 1997; Goff, Muntz et al. 1998). The use of a quantitative image analysis using the CAS 200 system has been favoured by some (Jordan, Kerns et al. 1993; Kerns, Jordan et al. 1994; Layfield, Saria et al. 1997; Anttila, Kosma et al. 1998).

With the CAS 200 system a two colour image analysis is used to calculate the proportion of Ki67 positive nuclei in relation to the total nuclear area. This type of image analysis measures absorbances of differentially stained cellular elements. Firstly, the observer determines the nuclear threshold on the specimen image field without positive stain, thus isolating nuclei from the image field. After that, the observer sets manually the antibody boundary, using the antibody threshold function. These nuclear and antibody thresholds are adjusted manually for every different sample to take account for different staining. The proportion of the Ki67 positive area in relation to the total nuclear area is then calculated on at least 10 different fields.

In analysis of association of Ki67 staining and ovarian cancer prognosis all studies have used a cut-off point to differentiate between 'high' and 'low' proliferation. Unfortunately the cut-off point varies from 7% in one study (Kerns, Jordan et al. 1994) up to 50% in another (Goff, Muntz et al. 1998). The reason for choice of cut-off is not well described in any study. The reproducibility of the scoring system used is also not commented upon in any study. Only one study states that the slides were scored blindly by the observer (Anttila, Kosma et al. 1998). In view of the above there have been differences found in the outcome of the studies.

However, most studies do show a significant correlation ($p<0.05$) on univariate analysis between 'high' Ki67 staining and poor overall survival (Jordan, Kerns et al. 1993; Kerns, Jordan et al. 1994; Altavilla, Marchetti et al. 1996; Layfield, Saria et al. 1997; Anttila, Kosma et al. 1998; Goff, Muntz et al. 1998). Only one study performed a multivariate analysis for survival including all the known clinical and

pathological prognostic factors for ovarian cancer and again showed a correlation of Ki67 with overall survival (p=0.04) (Anttila, Kosma et al. 1998). This study also showed an association of 'high' Ki67 staining and poor progression free survival on univariate (p=0.0024) but not multivariate analysis. In contrast one study of 104 patients showed no correlation of Ki67 staining with overall survival but this was the only study to use manually assessed groups for percentage staining of 0-20%; 21-50% and over 50 (Rohlke, Milde-Langosch et al. 1997).

Some studies have looked at the correlation of Ki67 staining with clinical and pathological prognostic factors in ovarian cancer. On the whole these have been negative for age (Layfield, Saria et al. 1997); grade of tumour and stage (Jordan, Kerns et al. 1993; Kerns, Jordan et al. 1994; Layfield, Saria et al. 1997). However only one study had a large number of patients (n=316) and did show an association of increased Ki67 staining and high grade (p=0.001) and increasing stage (p<0.001) but no association with age, histology or residual disease (Anttila, Kosma et al. 1998).

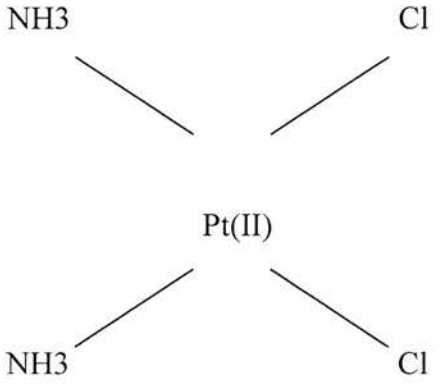
Two studies have examined the relationship of proliferation index as measured by Ki67 staining and response to chemotherapy by second look laparotomy results. They both showed no association (Layfield, Saria et al. 1997; Rohlke, Milde-Langosch et al. 1997).

3 (iii) - CISPLATIN CHEMOTHERAPY RESISTANCE

A - INTRODUCTION

Cisplatin is the mainstay of treatment in a number of human cancers, shown in figure 1. It was first noted by Rosenberg in 1965 that growth of *Escherichia coli* was inhibited in a continuous culture apparatus containing platinum electrodes. This was found to be due to *cis*-dichlorodiammineplatinum, hereafter called cisplatin. By 1973 it was clear that cisplatin had antitumour activity in a number of human cancers. Initial enthusiasm was dampened by renal toxicity until prehydration and prolonged infusion were shown to be an effective way of ameliorating the nephrotoxic side effects of cisplatin in 1977 (Rosenberg 1984).

Figure 1 - Chemical structure of cisplatin



Cisplatin preferentially forms intrastrand crosslinks with G-C rich regions of DNA (Stone, Kelman et al. 1974). Cisplatin forms a closed ring adduct with both N7 and O6 of guanine. The O6 position of guanine is normally involved in hydrogen bonding capacity to cytosine and this is blocked. On base pairing during cell replication the

guanine will then mispair with thymine if the adduct is not removed before replication occurs (Rosenberg 1984).

Since these studies it has been shown clinically that the amount of platinum-DNA adduct formation is directly related to the antitumour effects of cisplatin. In one phase I study of cisplatin and carboplatin in 24 different tumour types it was shown that the platinum-DNA adduct level measured by atomic absorbance spectroscopy (AAS) was directly related to disease response regardless of the tumour type ($p=0.0004$) (Reed, Parker et al. 1993).

B - MECHANISMS OF CISPLATIN RESISTANCE

Considerable effort has been invested in defining the cellular and molecular mechanisms responsible for resistance to cisplatin. Most published data are derived from preclinical model systems, where a large number of potential resistance mechanisms have been identified. However not all these mechanisms may be in important *in vivo*. The mechanisms identified can be broadly grouped into 2 categories;

- Resistance mechanisms that limit the extent of drug-induced damage
    - Altered accumulation
    - Thiols
    - DNA repair
- Resistance mechanisms that alter the cellular response to the damage that is induced
    - MMR proteins
    - HMG proteins
    - Apoptotic response

B (I) - Altered accumulation

The mechanism by which cisplatin enters cells is still not clearly defined. It is thought that it may enter cells via transmembrane channels. Decreased accumulation of cisplatin into cells is common in cell lines selected for cisplatin resistance *in vitro* (Gately and Howell 1993). However, this reduced accumulation does not generally correlate with the magnitude of resistance observed. Thus, it is probable that additional mechanisms are involved.

B (II) - Thiols

Cytosolic inactivation is another mechanism that can prevent cisplatin from reacting with intracellular target molecules. Intracellular sulphydryl compounds have been proposed to function in this manner (glutathione (GSH) and metallothionein (MT)). GSH covalently binds cisplatin at physiological concentrations (Dedon and Borch 1987) and can inhibit conversion of platinum-DNA monoadducts to potentially cytotoxic crosslinks (Eastman 1987). Linear correlations between GSH levels and cisplatin resistance have been reported in ovarian cancer cell lines and in human ovarian tumour biopsies (Perez 1998). In contrast, levels of glutathione S-transferase (GST), which are responsible for maintaining levels of GSH in cells, have been shown to be reduced in malignant ovarian tumours after chemotherapy with cisplatin (van der Zee, van Ommen et al. 1992).

Metallothionein is a low molecular weight, cystein rich metalloprotein that can react directly with cisplatin (Pattanaik, Bachowski et al. 1992). However, while metallothionein expression has been associated with cisplatin resistance in some models, no association has been seen in other models (Perez 1998).

B (III) - DNA repair

Nucleotide excision repair (NER) is important in the repair of bulky covalent lesions within the DNA, such as platinum-DNA lesions (Reed 1998). In human ovarian cancer patients it has been shown that high tumour tissue levels of ERCC1 mRNA (critically involved in NER) are associated with clinical resistance to platinum (Dabholkar, Bostick-Bruton et al. 1992). A large body of evidence suggests that NER is the predominant pathway that repairs platinum DNA adducts in cellular DNA (Reed 1998).

B (IV) - DNA damage tolerance and MMR activity

In a series of unrelated human ovarian carcinoma cell lines several potential resistance mechanisms to cisplatin were examined (Johnson, Laub et al. 1997). It was found that DNA damage tolerance was strongly correlated with cisplatin sensitivity, whereas no correlation was apparent for platinum accumulation, GSH levels, or platinum DNA adduct repair. This suggests that although DNA repair (by NER) is important quantitatively, it is DNA damage tolerance that is potentially important in determining cisplatin resistance. The mechanisms that confer tolerance to DNA damage are still

incompletely defined and are discussed in detail in the section on loss of MMR and cisplatin resistance.

B (V) - HMG proteins

The high-mobility-group (HMG) proteins are a multifunctional family of small non-histone chromatin-associated proteins. These proteins are involved in gene regulation and maintenance of chromatin structure. Several HMG-family proteins specifically recognise cisplatin-DNA adducts (Perez 1998). The product of the Ixr1/ORD1 gene, in yeast, is a HMG protein that specifically binds cisplatin-DNA adducts. Compared with parental strains Ixr1 deletion mutants contain fewer platinum-DNA adducts and are 2-fold resistant to cisplatin (Brown, Kellet et al. 1993). The full significance of HMG proteins in mammalian cells is not yet known.

B (VI) - Apoptotic response

The potential of altered capacity to undergo apoptosis in causing resistance to cisplatin is being increasingly studied. One critical regulator of apoptosis in response to cisplatin is p53 (Kastan, Onyekwere et al. 1991). Direct evidence for the role of p53 in cisplatin resistance comes from experiments using p53 genetic suppressor elements (GSE). By using GSE to cause a decrease in p53 protein levels and loss of p53 function, (including cell cycle arrest and apoptosis), there was up to an 8-fold increase in cisplatin resistance in the A2780 ovarian carcinoma cell lines transfected with GSE (Gallagher, Cairney et al. 1997).

Conclusions

A substantial number of mechanisms have been suggested to explain resistance to cisplatin chemotherapy. Many of these mechanisms rely on data from in vitro models and it is difficult to know how much influence they may have in clinical resistance in humans. However, an improved understanding of cisplatin resistance would be useful in a number of ways:

1. To predict clinical response to chemotherapy. This would allow prediction of patients requiring modulation of their therapy.

2. To identify novel targets for intervention that may improve the sensitivity to cisplatin.

Chapter 4 -
PATIENTS AND STATISTICS

4 (i) – PATIENTS FOR IMMUNOHISTOCHEMICAL ANALYSIS

All patients were selected from 3 previous clinical trials in ovarian cancer performed at the Beatson Oncology Centre, Glasgow since 1989, from whom histological blocks could be obtained. Data prospectively collected on these patients included; FIGO stage at diagnosis (International Federation of Gynaecology and Obstetrics) (FIGO 1987), performance status (PS); residual disease at primary operation (either <2cm or ≥2cm); histology; age; response to chemotherapy; time to progression and overall survival. All patients had given written informed consent for data collection as part of these trials, which were all approved by the local ethics committee. All patients had a histological diagnosis of ovarian adenocarcinoma and were treated with primary chemotherapy regimens containing cisplatin. In one trial patients were randomised to receive either high or low dose cisplatin (100 or 50 mg/m^2) respectively with 750 mg/m^2 cyclophosphamide (Kaye, Paul et al. 1996). In the second trial patients all received 100 mg/m^2 cisplatin and 750 mg/m^2 cyclophosphamide and were randomised to receive placebo or nimodipine (Cassidy, Paul et al. 1998). In the third trial all patients received 75mg/m^2 cisplatin and were randomised to receive Taxol 135mg/m^2 or cyclophosphamide 750mg/m^2 (Stuart, Bertelsen et al. 1998). Response to chemotherapy was assessed according to WHO criteria (Miller, Hoogstraten et al. 1981).

58 patients were included in the study. Their median age was 56 (range 27-71). Their other characteristics are shown in table 17. Fifteen patients are still alive with a median follow up of 94 months (range 32-112 months). Six of these 15 have progressed. For each patient all the available haematoxylin and eosin stained sections were reviewed and a representative block was selected for further study.

Table 17 - Patient characteristics

(N=58)		N=	%
Chemotherapy	Cisplatin (50)/Cyclophosphamide[1]	25	43
	Cisplatin (100)/Cyclophosphamide[1]	24	41
	Cisplatin (100)/Cyclophosphamide[2]	3	5
	Cisplatin (75)/ Taxol[3]	3	5
	Cisplatin (75)/ Cyclophosphamide[3]	3	5
Stage	IC	6	10
	II	9	16
	III	35	60
	IV	8	14
Performance Status	0	19	33
	1	34	59
	2	5	9
Residual Disease	<2cm (1)	20	34
	2cm or greater (2)	38	66
Histology	Serous cystadenocarcinoma	17	29
	Adenocarcinoma	13	22
	Papillary adenocarcinoma	19	33
	Mucinous cystadenocarcinoma	3	5

	Endometrioid carcinoma	3	5
	Clear cell carcinoma	2	3
	Unknown	1	2

References; 1 = (Kaye, Paul et al. 1996); 2 = (Cassidy, Paul et al. 1998); 3 = (Stuart, Bertelsen et al. 1998)

As part of the study 114 paraffin embedded blocks of tissue were sectioned and stained for the 4 antibodies; MLH1, MSH2, Ki67 and p53. 4 blocks had insufficient tumour material for scoring. Therefore 110 blocks were able to be scored. 13 blocks were repeats from the same patient at the same operation and are discussed in detail elsewhere. Therefore a total of 97 blocks were included in the study from a total of 63 patients.

Of the total 97 number of blocks, 58 were from chemonaive patients at their initial diagnostic laporotomy. Five patients had multiple post-chemotherapy operations and therefore the remaining 39 post-chemotherapy blocks were from 31 patients. In 5 patients with post-chemotherapy blocks, the original pre-chemotherapy specimens could not be recovered from the relevant pathology departments. Therefore pre- and post-chemotherapy paired samples are only available in 26 patients.

Out of an attempted 1166 slides only 880 slides were successfully stained and suitable for scoring i.e. a success rate of 75.5%. Failure of immunostaining was most often

due to lack of positive staining in the relevant control slide. In this situation the complete run was abandoned. The causes for variability in immunohistochemistry are numerous and attention to detail is the key. Timings must be accurate and the slides must not be allowed to dry out at any time.

4 (ii) – STATISTICAL METHODS

The associations between MMR, p53 and Ki67 scores were examined using Spearman's rank correlation coefficient (Conover 1980). The association of these scores with stage and histology were examined using Kruskal-Wallis one-way analysis of variance (Conover 1980) (this corresponds to the Mann-Whitney U test when there are only two groups).

Survival and progression times were measured from the date the patients were recruited into the respective trials. For the survival analyses only cancer deaths were considered as events, deaths from other causes were treated as censoring events. The Cox proportional hazards model (Parmar and Machin 1995), stratified for type of chemotherapy, was used to analyse these end-points. MMR, p53 and Ki67 scores were examined individually and then in multivariate models including the established prognostic factors of stage, extent of residual disease and performance status. P-values for hazard ratios are from the associated likelihood ratio test. The Kaplan-Meier method (Parmar and Machin 1995) was used for the survival plots.

Response data (complete/partial versus stable/progression) was examined using logistic regression (Armitage and Berry 1987). The type of chemotherapy was included in all the logistic regression models. MMR, p53 and Ki67 scores were first examined individually and then in models containing the three prognostic factors. P-values for odds ratios are from the associated likelihood ratio test.

Weighted kappa (Fleiss and Cohen 1973) values were used for measuring inter- and intra-observer agreement on MMR and p53 scores. The intra-observer agreement for Ki-67 scores was measured using an intra-class correlation coefficient. The inter-observer agreement was obtained by comparing the two observers' scores for the same slide. The intra-observer agreement was measured by comparing the same observer's scores on the same slide on two different occasions at least 6 months apart. The intra-slide agreement was measured by comparing the same observer's scores on different slides from the same patient.

All P-values quoted are two-tailed.

Chapter 5 – METHODS AND SCORING FOR IMMUNOHISTOCHEMISTRY

1 - IMMUNOHISTOCHEMISTRY

1A - IMMUNOHISTOCHEMISTRY TECHNIQUE

I. Introduction

The loss of function in any of four human DNA mismatch repair genes, (hMSH2, hMLH1, hPMS1 and hPMS2) is thought to lead to deficient mismatch repair of DNA in the somatic cells leading to increased mutations and thereby cancer development. Microsatellite instability (MSI) detected by PCR analysis has to date been the hallmark of loss of the function of these genes. Western and Northern blotting has confirmed the associtation of loss of mismatch repair (MMR) protein expression with microsatellite instability. However, this technique relies on fresh samples for DNA extraction and requires a normal tissue sample. Expression of MMR proteins can now be examined in both fresh and archival paraffin embedded tissue samples using immunohistochemical techniques.

II. Streptavidin-biotin immunoperoxidase method

Immunohistochemistry has undergone numerous refinements over the past 20 years and is now routinely performed for both diagnostic and prognostic information on patient tissue samples in pathology laboratories. Numerous kits are available commercially and choice is normally based on local experience and the cost of the

monoclonal antibodies used. The technique used below employs the streptavidin-biotin technique coupled to peroxidase activity for use as a label (Hsu, Raine et al. 1981). This relies on the markedly high affinity of avidin, a 68, 000 mw glycoprotein, for the small molecular weight vitamin, biotin. Because this affinity is over one million times higher than that of antibody for most antigens, the binding of avidin to biotin is essentially irreversible. In addition to this high affinity, the biotin/avidin system can be effectively exploited because avidin has four binding sites for biotin and most proteins (including antibodies and enzymes) can be conjugated with several molecules of biotin. These properties provide the potential for macromolecular complexes to be formed between avidin and biotinylated enzymes.

A small amount of unlabelled primary antibody when bound to the antigen of interest in the tissue then binds a biotinylated secondary antibody and subsequently a preformed avidin and biotinylated horseradish peroxidase macromolecular complex. Diaminobenzidine (DAB) is then used as a substrate for the bound antibody complex that produces a brown colour after undergoing oxidation. If no primary antibody is bound to the tissue there will be no peroxidase activity and the tissue will not stain brown with DAB. In order to visualise negative tissue samples they are counterstained with a routine haematoxylin stain (blue). The method below is divided into several steps for clarity. The whole procedure should take approximately 6 hours for 20 slides.

This technique was shown to be superior to the previously used PAP (peroxidase-antiperoxidase) method and allowed the use of antibodies diluted 20 to 40 times. The

use of low concentrations of primary antibodies minimises background staining and increases method-specificity.

III. ***Antigen retrieval***

At the time of starting immunohistochemical analysis for MMR proteins as part of this study there had been no publication on the use of antibodies for staining paraffin embedded material and a therefore it was necessary to develop a protocol. Microwaving as a technique for exposing antigens to the antibodies has been developed over the last 14 years (Leong 1996) and was known to be a good technique for both p53 and Ki67 immunohistochemistry. Tissue sections fixed in formaldehyde and then embedded in paraffin often show a reduction in and sometimes loss of immunoreactivity for many antigens. This is thought to be due to cross-linking of reactive sites within the same protein and between different proteins via methylene bridges. Calcium ions may also play a role. Attempts to unmask antigens have often been based on pre-treatment of sections with proteolytic enzymes e.g. trypsin. (See review by (McNicol and Richmond 1998)). Shi et al. showed that the use of microwave heating improved the staining in 39 of 52 antibodies studied (Shi, Key et al. 1991). The energy from heating disrupts the protein-protein crosslinks and may also break down links between calcium ions and amino acids. Heating can be achieved by various methods including microwave, water bath, wet autoclaving or pressure cooker. There has been a suggestion by one group that autoclaving is to be preferred to microwave as they showed a consistent loss of morphology (swollen cells and reduced mitotic index; $p<0.001$) on slides subjected to microwave compared with autoclaving or no heating (Hunt, Attanoos et al. 1996). However, the study was only on 6 cases of non-Hodgkin's B cell lymphoma and has not been repeated in other tissues. Therefore the heating method chosen often depends on local availability and

experience. The slides must be heated in aqueous solutions and the pH of the buffer is often of importance (Shi, Imam et al. 1995). The best results have been reported using citrate buffer at pH 6.0 or Tris-HCl at pH7-8. For p53 and Ki67 the optimum retrieval strategy is the use of microwave heating and citrate buffer at pH 6.0 (Taylor, Shi et al. 1994).

Varying lengths of time of microwaving were used to optimize positive staining and reduce any false positive staining or loss of material from the slide. Other techniques available to encouraging binding of the primary antibody to the slide include overnight incubation; 0.05% saponin incubation for 30 minutes and 0.1% trypsin incubation at 37 C for 30 minutes. Results of these different techniques are shown in table 18.

IV. ***Primary antibodies used***

As no technique was previously described for MMR protein immunohistochemistry on paraffin embedded material, varying dilutions of antibody were used to optimize positive staining and reduce background staining with all the antibodies used. Results are shown in table 19. Each optimization was repeated at least twice until reproducible results were obtained. Marked variability with both false positive and negative staining was noted with PMS2 despite 9 separate attempts to optimize conditions. Of the 215 tumour slides stained for PMS2 in 13 separate runs only 50 slides (23%) could be scored from 4 of the runs. In view of the unpredictability of the staining, PMS2 was excluded from the antibodies reported in the final results.

A. Anti-hMLH1. Clone G168-15. PharMingen®. This anti-human mouse antibody was purified from hybridoma tissue culture supernatant by Protein G affinity chromatography. Full-length human recombinant MLH1 was expressed as a maltose binding-MLH1 fusion protein, affinity purified and used as immunogen. The resulting antibody recognizes human and mouse MLH1 (Baker, Plug et al. 1996).

B. Anti-hMSH2. Clone FE11. Calbiochem®. This anti-human mouse monoclonal antibody was generated by immunizing BALB/c mice with a carboxyterminal fragment of the human MSH2 protein and fusing with SP2/O mouse myeloma cells.

C. Anti-hPMS2. Clone 9. Calbiochem®. This anti-human mouse monoclonal antibody was generated by immunizing CB6 F1 mice with full length PMS2 protein expressed in E. coli and fusing with SP2/O mouse myeloma cells. The epitope is unknown.

D. Anti-p53. Clone DO-1. Calbiochem®. This anti-human mouse monoclonal antibody recognises an epitope on residues 21-25 of human p53. It recognises both wild type and mutant human p53. It was generated by immunizing BALB/c mice with recombinant human wild type p53 protein and fusing splenocytes with mouse myeloma cells (Vojtesek 1992).

E. Anti-Ki67. This is a polyclonal antibody developed from rabbits immunised with a synthetic peptide deduced from the human cDNA

sequence coding for the Ki67 antigen. It detects the native as well as recombinant parts of the human Ki67 antigen in different test systems. It has the advantage of being able to detect the Ki67 antigen in routinely processed, paraffin wax embedded material. (Key, Peterson et al. 1993). The Ki67 antigen is present in G_1, S, G_2 and M phases of the cell cycle but is absent in G_0. Because of this Ki67 is a good estimation of the growth fraction as it is only present in proliferating cells and is absent in quiescent cells (Gerdes, Li et al. 1991). As this is a rabbit antibody it can be used for double labelling experiments with mouse antibodies.

V. ***Materials*** Using Vectastain® *Elite* ABC Kit (See Note 4)

A. Stable Solutions for immunostaining: All stable at 20°C.

1. Phosphate Buffered Saline (PBS)

2. 0.1% hydrogen peroxide (per 1 litre): 1 ml 100 vol. hydrogen peroxide + 1 litre distilled water

3. 10mM Sodium tricitrate buffer (per 1 litre): 2.94g sodium tricitrate + 1 litre distilled water. Adjust to pH 6 with HCl

4. Antidote to DAB (per 100 mls): 3g potassium permanganate ($KMn0_4$) + 2g sodium carbonate (Na_2CO_3) + 100 mls distilled water

B. Unstable Solutions for immunostaining: Keep on ice and make up fresh. Solutions stain approximately 20 slides (See Note 5).

1. Blocking Serum (Vectastain® Yellow - 10mls): 10 mls PBS + 150 µl Normal Horse Serum (i.e. 3 drops yellow block)

2. Biotinylated Antibody (Vectastain® Blue - 10mls): 10 mls PBS + 150 µl Normal Horse Serum (i.e. 3 drops yellow block) + 50 µl biotinylated anti-mouse IgG (i.e. 1 drop blue block). Note for Ki67 use biotinylated anti-rabbit IgG

3. ABC reagent (Vectastain® Grey). ***Make up 30 minutes before use***. 5 mls PBS + 2 drops solution A + 2 drops solution B

4. DAB diaminobenzidine tetrahydrochloride (per 5mls): 5 mls distilled water + 2 drops Vectastain® buffer + 2 drops hydrogen peroxide + 4 drops Vectastain® DAB reagent. Note - possible carcinogen - use gloves

C. Primary antibodies: (per 2 mls = approx. 20 slides), (See Note 6+7)

1. Anti-hMLH1: 20 µl MLH1 antibody (500 µg/ml purified anti-human mouse monoclonal IgG, clone G168-15, PharMingen®, stock solution kept at 4°C) + 1980 µl PBS. i.e. 5 µg/ml solution or 1 in 100 dilution

2. Anti-hMSH2: 20 µl MSH2 antibody (100 µg/ml purified anti-human mouse monoclonal IgG, Ab-2 clone FE11, Calbiochem® stock solution kept at 4°C) + 1980 µl PBS. i.e. 1 µg/ml solution or 1 in 100 dilution

3. Anti-hPMS2: 40 µl PMS2 antibody (100 µg/ml purified anti-human mouse monoclonal IgG, Ab-1 clone 9, Calbiochem® stock solution kept at 4°C) + 1960 µl PBS. i.e. 2 µg/ml solution or 1 in 50 dilution

4. Anti-Ki67: 10 µl Ki67 antibody (250 µg /ml purified anti-human rabbit polyclonal IgG, Dako® stock solution kept at 4°C) + 1990 µl PBS. i.e. 5 µg/ml solution or 1 in 50 dilution

5. Anti-p53: 40 µl p53 antibody (100 µg/ml purified anti-human mouse monoclonal IgG, Ab-6, Oncogene® stock solution kept at 4°C) + 1960 µl PBS. i.e. 2 µg/ml solution or 1 in 50 dilution

D. Solutions for counterstaining:

1. Haematoxylin: Harris formula filtered before use, Surgipath®

2. 100% ethanol

3. 70% ethanol

4. Histo-clear®, Fischer Scientific Ltd.

5. Scott's Tap Water: 1 in 10 dilution in distilled water of Surgipath® Scott's tap water substitute. Warning: can cause skin irritation - use gloves

6. Acid Alcohol: 10 mls concentrated hydrogen chloride (specific gravity 1.16 g/ml - approx. 33%) in 990 mls 70% ethanol

7. Hystomount®, Hughes and Hughes Ltd.

VI. ***Method***

A. Dewaxing of slides. In a laminar flow hood (See Note 8).

1. Place slides in metal rack (maximum 20). Wash in bath of Histo-clear for 20 minutes

2. Rinse in bath of 100% ethanol for 1 minute (See Note 9)

3. Rinse in bath of 70% ethanol for 1 minute

4. Rinse in tap water for 1 minute

5. Wash in bath of PBS for 5 minutes (See Note 10)

B. Removal of endogenous peroxidase activity (See Note 11)

1. Wash in bath of 0.1% hydrogen peroxide for 20 minutes

2. Wash in bath of PBS for 5 minutes

C. Antigen retrieval (See Note 12)

1. Fill microwave box with 750 mls of sodium tricitrate buffer (See Note 13)

2. Place slides in plastic rack and place rack into box, cover with cling film, pierce 3 times

3. Microwave on full power (650W microwave) for 15 minutes (See Note 14)

4. Leave to cool for 20 minutes

D. Isolate the tissue section (See Note 15)

1. Wipe each slide in turn around tissue section with tissue paper to dryness.

2. Circle tissue section with 5mm margin with PAP-pen

E. Vectastain® ABC technique

1. Fill bottom of immunostaining box with damp tissue paper (See Note 16). Place slides one at a time into immunostaining box. Cover tissue section with 3-5 drops (approx. 100-200 μl) of yellow block and leave for 20 minutes

2. Tap off yellow block (blocking antibody), one slide at a time and cover tissue section with 100 μl, (2-3 drops) of primary antibody of interest (i.e. hMLH1, hMSH2, or hPMS2). Leave for 30 minutes

3. Place slides in metal rack and wash in PBS for 5 minutes

4. Place slides into immunostaining box and cover tissue section with 3-5 drops (100-200 μl) of blue block (biotinylated antibody). Make up ABC solution at the point and leave for 30 minutes

5. Place slides in metal rack and wash in PBS for 5 minutes

6. Place slides in immunobox and cover tissue section with 3-5 drops (100-200 µl) of grey block (ABC reagent). Leave for 30 minutes

7. Place slides in metal rack and wash in fresh PBS for 5 minutes

8. Place slides in immunobox and cover tissue section with 3-5 (100-200 µl) drops of DAB (See Note 17). Leave for 10 minutes

9. Place slides in metal rack and wash in PBS for 5 minutes

10. Use 5 mls of antidote to DAB over the immunobox

F. Counterstain with haematoxylin. In a laminar flow hood (See Note 8).

1. Rinse in tap water

2. Wash in haematoxylin for 60 seconds (See Note 18) and then rinse in tap water

3. Rinse in acid alcohol for 2-10 seconds (See Note 18) and then rinse in tap water

4. Wash in Scott's tap water for 60 seconds and then rinse in tap water

5. Wash in 70% alcohol for 60 seconds

6. Wash in 100% alcohol for 60 seconds

7. Place in Histo-clear® for at least 5 minutes

G. Mount slides (Perform in laminar air flow hood)

1. Place 20 coverslips on filter paper and place a drop of Hystomount® on each coverslip (See Note 19)

2. Take each slide in turn and wipe off excess Histo-clear® around tissue section. Place tissue section face down onto coverslip and leave for 10 seconds

3. Invert slide and coverslip so that the coverslip is face up and leave to set for at least one hour

VII. Notes

1) Tissue sections will be brown if the protein is present (positive) and blue if negative. It is essential for each run to include a known positive and negative slide control. We used sectioned paraffin embedded samples of cell lines with known mismatch repair protein status, i.e. Ovarian A2780 as a positive control for MLH1, PMS2 and MSH2, Ovarian A2780/CP70 for hMLH1 and hPMS2 negative (Anthoney, McIlwrath et al. 1996) and Colon LOVO 1 as negative for hMSH2 (Umar, Boyer et al. 1994).

2) To immunostain fresh fixed samples omit step one and place directly into PBS. Start with removal of endogenous peroxidase activity (step 2 in method).

3) Be gentle with the slides. The tissue sections can wash off if moved in solutions too vigorously. Using APES (Sigma) coated slides to mount the tissue sections will help them to stick.

4) The use of the Vectastain® *Elite* ABC kit is a personal choice. It does allow a small amount of expensive antibody to be used for a positive result.

5) Vortex all solutions before using to ensure even mixing.

6) Different batches of primary antibody give variable staining. With a new batch it is a good idea to run positive and negative controls with dilutions suggested but also 50% and 200% of these. We have found hPMS2 in particular to vary and require different dilutions depending on the batch.

7) To prevent variability of staining try to keep the stock solutions of primary antibody on ice at all time and return to storage as promptly as possible.

8) Histo-clear® and Hystomount® give off noxious fumes. Perform all steps involving these in a laminar flow hood.

9) Check the slides when in 100% ethanol at step 1c for dewaxing. If they are adequately dewaxed the section will be the shape of the tissue section. If they are not adequately dewaxed the section will still be the square shape of the original paraffin block. If this is the case place back into the Histo-clear® for another 10 minutes and check again. Better to check at this stage than to perform the whole immunostain and find out at the end it has not worked due to persistent wax!

10) When washing in PBS place the metal rack with the slides into a glass bath containing enough PBS to cover the tops of the slides (usually 350mls). Place the

bath on a rocker table for the 5 minutes to ensure even washing. Change the PBS after each wash ideally, but certainly after the peroxide step and the grey block to prevent false positive staining.

11) All tissue sections of interest must be checked for endogenous peroxidase activity (which will give a false positive result). Normally the 0.1% hydrogen peroxidase step should solve this problem but it is worthwhile checking each block by running a slide through the whole immunostain leaving out the primary antibody step. This slide should be negative (blue) if there is no endogenous activity.

12) We have tried different forms of antigen retrieval with saponin, trypsin and different times of microwaving and found 15 minutes in a microwave to give the most consistent results.

13) Make sure there is enough buffer in the microwave box so that the tops of the slides are covered even after 15 minutes of boiling. If this is a problem, perform the microwaving in 3 separate 5-minute steps and top up with buffer after each 5 minutes. If the slides do dry out there will be brown 'hot spots' as artifact on the final slides. Use a microwave with a turntable to prevent 'hot spots' and place the box eccentrically on the table, not in the middle.

14) Watch yourself with the microwave step. The buffer is boiling hot after 15 minutes in the microwave. Use protected oven-gloves to remove the box from the microwave and leave the cling film on until it is cooler.

15) The PAP-pen step allows you to use smaller amounts of primary antibody and prevents drying out of the tissue sections. After each step in the immunobox

double check each slide to make sure the solution is up to the edge of the circle drawn with the PAP-pen.

16) It is critical to consistent immunostaining not to allow the tissue section on the slide to dry out at any stage. This entails covering each slide with the next solution on a one by one basis. Do not be tempted to take all 20 slides out of the bath, place into the immunobox, and then cover with solutions. They will dry out giving artifact staining on the final slide. Ensure the immunobox is kept humid by lining the bottom with tissue paper soaked in warm water and cover the box with a plastic lid during the incubation times for the slides.

17) DAB is a potential carcinogen. Always use gloves when handling and soak all equipment which touches DAB in an equal amount of antidote afterwards for at least 30 minutes.

18) The depth of counterstain is of personal choice. The slides will be made bluer by leaving in haematoxylin for longer (step 6b) or leaving in acid alcohol, which leaches out the colour (step6d), for a shorter time.

19) Using too much Hystomount® will make the final slides messy to handle. Using too little might allow the tissue section to dry out with time. The ideal amount spreads from a central drop to cover the square of the coverslip in about 10 seconds. The amount to use will vary with the size of the coverslip. Always choose a coverslip size that adequately covers the tissue section.

20) When the slides are prepared from the paraffin embedded material they loose antigenicity and will stain false negative (blue) over a period of months, if stored

at room temperature (Jacobs, Prioleau et al. 1996). Store prepared slides at 4°C prior to immunostaining.

21) Scoring the immunostain is a matter of personal preference. It is usual for each slide to be scored for both the intensity of the stain and the % of cells stained. Automated systems e.g. CAS system, exist for automated counting of the % of cells stained. It is good practice for the slides to be independently scored by at least 2 observers, blinded to any clinical information. Inter-observer and intra-observer variation should be calculated aiming kappa scores above 0.5.

Table 18 - Results of different methods of antigen retrieval for MLH1 antibody

METHOD OF ANTIGEN RETRIEVAL	A2780 (+VE)	CP70 (-VE)
None	-	-
Overnight incubation	weak +	-
0.05% saponin	-	-
0.1% trypsin	-	-
4x5 mins microwave in 200 mls citrate buffer	*	*
2x5 mins microwave in 200 mls citrate buffer	*	*
2x5 mins microwave in 750 mls citrate buffer	-	-
4x5 mins microwave in 750 mls citrate buffer	+++	False ++
3x5 mins microwave in 750 mls citrate buffer	+++	-

*= Sections 'cooked' off slide; ++ = moderate staining; +++ = good staining

Ovarian cancer cell lines; A2780 = MLH1 normal; CP70 = MLH1 defective

Table 19 - Results of different antibody dilutions

MLH1 Dilutions	A2780 (+VE)	CP70 (-VE)
1 in 10	+++	+++
1 in 20	+++	+++
1 in 50	+++	+
1 in 100	+++	-
1 in 200	++	-
1 in 500	weak +	-
PMS2 Dilutions	A2780 (+VE)	CP70 (-VE)
1 in 5	+++	+++
1 in 20	+++	+/-
1 in 50	Variable from weak + to +++	Intermittent +ve
1 in 100	Weak +	-
1 in 200	Weak +	-
MSH2 Dilutions	A2780 (+VE)	LOVO (-VE)
1 in 20	+++	+
1 in 50	+++	+/-
1 in 100	+++	-
1 in 200	+	-

Ki67 Dilutions	Normal Colonic Crypts Of Lieberkuhn (+VE)	Normal Colonic Epithelium(-VE)
1 in 50	+++	++
1 in 100	+++	+
1 in 200	+++	-
p53 Dilutions	Normal Colon Epithelium (-VE)	Cp70 (Mutant P53, Strongly +++VE)
1 in 20	++++	++++
1 in 50	-	++++
1 in 100	-	++++
1 in 200	-	++++

- = no staining; + = weak staining; ++ = moderate staining; +++ = good staining; ++++ = strong staining.

1B - IMMUNOHISTOCHEMISTRY – Immuno Reactive Score or Histoscore

The reporting of immunohistochemistry results has been a matter of controversy for many years. Most work on this area has been done on oestrogen receptor (ER) analysis in breast carcinoma, which is a known prognostic factor. Several authors in the 1980s developed an *'immuno reactive score'* (IRS) or *'histoscore'* which is based on a score for both intensity (I) and percentage of cells (%). However the mode of calculation of differs markedly. Firstly, there is no agreement on the number of categories for intensity to be used (0-3 or 0-4). Secondly, the percentage of positive cells is sometimes on a subjective, often non-linear scale (0-3), whereas in others it is the actual percentage, often rounded in decades, of cells positive for each intensity category. Thirdly, the calculation of the final overall score varies from simple addition (I + %) (Reiner, Neumeister et al. 1990), to multiplication (I x %) (Remmele, Hildebrand et al. 1986), to complex formulae $\{\Sigma P_i x(i+1)\}$ (McCarty, Miller et al. 1985). Interestingly all of the above have been shown to be predictive for overall survival of patients e.g. (Reiner, Neumeister et al. 1990) (additive), (Kieback, Press et al. 1993) (multiplication), (Kinsel, Szabo et al. 1989) (complex).

The most commonly used 'H-score' gives a score from 0 to 300 and is calculated as follows (McClelland, Finlay et al. 1990):

H-score = (% of cells stained at intensity category 1 x 1) + (% of cells stained at intensity category 2 x 2) + (% of cells stained at intensity category 3 x 3)

An early study on simple scoring systems by Remmele et al. ER detection by the gold standard biochemical assay of dextran-coated charcoal (DCC) was compared to immunohistochemistry (Remmele, Hildebrand et al. 1986). In 156 breast cancer samples they found a closer correlation of biochemical and immunohistochemistry scores using a histoscore than just the intensity or percentage scores alone. They also commented on a closer correlation between DCC and the histoscore if it was multiplied (I x %) compared to added (I + %); r=0.5947 and 0.5372 respectively. However, they gave no p value or confidence intervals for this difference.

In another study of ER in breast cancer three methods of scoring immunohistochemistry were compared: (1) an additive (I+%) *quickscore;* (2) a multiplicative (Ix%) *quickscore*; and (3) the time consuming *H-score* (as detailed above; range 0 to 300) (Detre, Jotti et al. 1995). In 96 tumours they found that the *quickscores* (either additive or multiplicative) gave as good a correlation as the *H-score* with the gold standard enzyme immunoassay (EIA), shown in table 20. They also showed the *quickscores* were closely correlated to the *H-score* but importantly took only approximately a quarter of the time to measure. They showed no advantage between the additive and the multiplicative *quickscore* with the two being very closely related (r=0.994). They also confirmed that the *quickscore* was reproducible, with two independent observers in total agreement in 73% of cases. They suggested the multiplication score might be preferred as it gives a wider range of results. The major criticism of the multiplication histoscore is that it places a large reliance on the intensity score, I, which is known to show intra- and inter-observer variation.

Table 20 – Comparison of additive, multiplicative quickscore and histoscore

Detre et al Score	Compared to	Spearman rank correlation coefficient (r)
H-score	EIA	0.843
I x % quickscore	EIA	0.831
I + % quickscore	EIA	0.832
I + % quickscore	I x % quickscore	0.994
I x % quickscore	H-score	0.892
I + % quickscore	H-score	0.887

Ravn et al. examined the reproducibility of subjective categories for both the intensity and percentage of cells staining for ER and PgR in 87 endometrial biopsies (both stromal and epithelial cells) (Ravn, Rasmussen et al. 1993). For intensity no adequate distinction between categories 1 and 2 could be made by subjective estimation (inter-observer and intra-observer kappa scores <0.5). However strong staining (category 3) or absence of staining (category 0) was reproducible (inter-observer and intra-observer kappa scores >0.5). Kappa scores are explained in section 2B of results chapter 4.

For percentage scoring they showed good agreement for the 0-10% and >90% categories (inter-observer and intra-observer kappa >0.5). By contrast there was poor

agreement (inter-observer and intra-observer kappa <0.5) for the 4 categories from 10-90% (i.e.10-25; 25-50; 50-75; 75-90%). By reducing the percentage categories (0-10; 10-50; 50-90; >90%) to only four groups (0-3) they were able to produce a more reproducible scoring system (inter-observer and intra-observer kappa >0.5). They compared this 4 category, %-score to a time consuming absolute count of cells (number stained/total number of cells) performed at high magnification with a counting frame. For epithelial cells there was a good agreement between the two systems (kappa 0.59) but not for stromal cells (kappa<0.5). Interestingly they showed no advantage to a simplified H-score, based on (I x %)/3, compared to %-score (0 to 3) alone for correlation to the gold standard EIA measurement (r=0.659 and 0.674 respectively for ER). They did not compare intensity score with either the %-score or H-score.

The Gynaecological Cancer Co-operative Group of the European Organisation for Research and Treatment of Cancer (EORTC) published guidelines in 1997 on a scoring system for immunohistochemical staining in basic research (van Diest, van Dam et al. 1997). With regard to the histoscore system they commented that (1) the percentage scores are only rough estimates without counting; (2) an intensity score assigned to an individual nucleus is highly subjective and (3) there are many inter-observer discrepancies in mid-range scores (van Diest, Weger et al. 1996). They recommended the use of an interactive approach based on stereology that is quick, simple, and reproducible (Polkowski, Meijer et al. 1996; Oudejans, Jiwa et al. 1997). This approach applies a systematic random sampling tehnique for selection of a defined number of fields of vision in which the positivity of cells is counted with a

point grid. A few cells (2-4) are counted in a large number of fields (50-100). However, the Polkowski study (Polkowski, Meijer et al. 1996) can be criticised as the 32 areas studied (from 25 cases of Barrett's oesophagus) were carefully pre-selected and reproducibility studies were performed on only 8 areas. Similarly the study by Oudejans on 80 cases of Hodgkin's disease does not give any reproducibility results (Oudejans, Jiwa et al. 1997). This relatively new method of scoring has not yet been applied to many studies in contrast to the histoscore method. It has yet to be accepted as the gold standard. They do recommend that regardless of the system used, the quantification method should be provided, in addition to the exact data.

Kappa scores

It is of concern that few studies of the biological significance of immunohistochemistry produce inter and intra-observer agreement measurements. Adams et al. recently reported on a comparison of a 4 point intensity scale (0-3) with a descriptive scale which combined both the intensity and distribution of positive staining (Adams, Green et al. 1999). In scoring 92 cervical tumours for p53 and EGFR twice, 6 months apart, by three independent observers they showed a very low value for the intra-observer variation with a mean weighted kappa of 0.32 (range 0.24 to 0.47). There was no large difference in inter-observer kappa variation between the 2 scoring systems with an overall mean weighted kappa score of 0.49 for the 4 point intensity scale compared to an overall mean weighted kappa score of 0.48 for the descriptive method. However, a marked improvement in weighted kappa was found when the more widely varying scores were discussed by the panel of observers and a consensus score agreed (k=0.63).

The degree of staining of a slide is very subjective. Many different systems exist for the scoring of immunohistochemical stains. Any system should be a) sensitive enough to identify important differences and b) reproducible in time and between different scorers. All scoring should be of the tumour cells alone and not of surrounding normal tissue.

1B (I) - The %-score

Slides can be scored for the number of positive cells. The most frequently used technique is to score the percentage of cells positive in decades i.e. 10%, 20%, 30%, etc. This has the advantage of a linear scale but has the disadvantage of the difficulty in the naked eye being able to tell differences of only 10%. The large interobserver and intraobserver variation with this technique can result in any difference found being spurious. To try to improve utility we opted for an easier percentage score that we felt was obvious to the naked eye, shown in table 21. Our scale has the disadvantage of loss of detail with a score of 2 and nonlinearity.

Table 21 – Percentage score of cells

SCORING % OF CELLS	
0	less than 5% of cells positive
1	5 to 20% of cells positive
2	20 to 80% of cells positive
3	over 80% of cells positive

This can be said in lay terms as follows,

0- very rare cells stain positive

1- a few cells positive but most negative

2-moderate numbers of cells positive but noticeable amount still negative

3- most cells positive with very rare negative cells

1B (II) - The I-score

Intensity of staining is a reflection of the gene product. A cell with a large amount of the protein of interest will turn a much darker brown compared to a cell with only a few copies of the protein. Immunohistochemical detection of protein depends on the sensitivity of the antibody. Any variability in the time each of the antibodies is left in contact with the slide and any variability in the concentration of solutions will result in a variation in intensity of the stain. Therefore to make any score for intensity relies on having an internal positive and negative control for each run.

Table 22 – Scoring for intensity

SCORING FOR INTENSITY	
0	no stain
1	weakly positive (+)
2	Positive (++)
3	strongly positive (+++)

1B (III) - The IHC-score

The overall score for each slide was calculated by addition of the % and I score i.e. a scale from 0 to 6 maximum. This had the advantage of including both types of scoring but had the disadvantage of giving equal weight to both scores. Previous scoring has included a multiplication of the decade % of cells by the intensity to score to try to give a 'product' of the total amount of protein of interest on the slide.

1B (IV) - Sources of variation

Variability in the scoring can be due to a number of factors including; contamination by normal cells; necrotic tumour cells; inter-scorer variability; intra-scorer variability; variability in the staining technique. We have tried to allow for variability in the staining technique by scoring 2 slides for each block of interest. To allow for interobserver variability each slide has been scored independently by 2 observers. We have included some repeat scores to measure intraobserver variability. All slides were scored blinded to clinical outcome to avoid potential bias.

1C - IMMUNOHISTOCHEMISTY- Ki67 SCORING

Unlike the other primary antibodies being studied we decided to use image analysis for scoring the Ki67 slides for 2 reasons, viz:

a) There appeared to be no variation in intensity over the slides. There was either a strong brown stain in a cell or none.

b) Much previous work has been done on scoring for Ki67 using image analysis.

2 slides were scored for each block of interest using CAS 200® system. The percentage of positive nuclear staining was calculated using the Quantitative Proliferation Index® program. The tissue to be analyzed is stained so that non-specific nuclear proteins (and thus, morphologically, the entire nucleus) are stained with haematoxylin; and specific nuclear proteins (e.g. Ki67) are immunostained with diaminobenzidene (DAB). These two components of the cell nucleus can then be sensed separately by the two image sensing channels of the CAS 200® camera at 620nm and 500nm respectively. All of the nuclei will be sensed at 620nm. However, only the antigen antibody stain complex absorbs light at 500nm, since haematoxylin is transparent at this wavelength. Stereological measurement principles are then used to estimate the percentage, by volume, of antibody-tagged nuclei as a ratio of the total volume of cell nuclei. On each image field a measurement is made of the total area of the nuclei from the 620nm image, and the total area of the antibody-tagged nuclei in the 500nm image. The latter divided by the former, expressed as a percentage, is the proliferation index. Individual cells are not counted but to correct for this average nuclear size and antibody size is estimated for each slide. For each slide the final percentage score was the mean of at least 10 fields and at least 1,000 cells.

2 – VARIABILITY OF IMMUNOSCORES

2A - *SOURCES OF VARIATION*

Variability may exist within the immunoscores for a number of reasons. The principle variability we are trying to measure is the variability due to the expression of the protein in question. However other sources of variation exist and may lead to bias of the results. These include;

1) *Variability due to the staining technique*. This can be determined by staining the same tissue section on multiple staining runs.

2) *Variability due to the observer over time*. This can be examined by scoring the same slide on different occasions over time.

3) *Variability between observers*. This can be investigated by 2 observers who independently score the same slide. It is important that the 2 observers use the same agreed scoring system.

4) *Variability due to personal bias of the observer*. Immunohistochemistry scoring attempts to assign a score to a subjective assessment of browness and percentage of cells staining. It is open to bias from preconceived ideas of the likely results by the observers. To avoid bias from this, all slides should be scored blinded to clinical outcome.

5) *Variability of protein expression* between tissues and within the same tissue.

For MLH1, MSH2 and p53 scoring in order to reduce variability due to observers (3) in this study we had two independent examiners; Dr M Mackean (MJM) and Dr. D Millan (DM). DM is a consultant histopathologist specializing in gynaecological malignancy. DM and MJM agreed a scoring system as previously described in the materials and methods. DM provided training for MJM over a period of 4 months in identifying ovarian tumour cells and the scoring system. All subsequent scoring included in the results section was scored independently. Both scorers were blinded to clinical outcome thus reducing variability due to beliefs of the observers (4). We have called this the ***inter-observer*** variation.

To reduce variability due to the staining technique (1) both the examiners scored two separate slides per tissue. These slides were stained on different runs at least 24 hours apart. As two slides from the same tissue were scored at different examinations the variability in those scores included both variability due to time (2) and due to staining technique (1). We have called this the ***intra-slide*** variation.

MJM scored 46 slides a second time after 6 months from the first scoring blinded to the first scores. This assessed the degree of variability over time (2). We have called this the ***intra-observer*** variation.

Variability of tissue expression was examined in multiple blocks taken at the same operation described in results section 1c. We have called this the ***intra-block*** variation.

2A (I) - Reporting of variability

There are a number of methods used to examine the variability of scoring systems. The most accurate statistical method which gives a weighting to the difference in the score (e.g. a difference of 2 affects the score more heavily than a difference of 1) is to use the kappa score. However in the literature an often reported method is to give the percentage of times the score varied, and by how much, between the 2 scores. The results below are reported in both formats for completeness. The use of a correlation coefficient between scores, as is sometimes used, is inappropriate because it does not take into account the degree of agreement by chance alone.

2A (II) - The principle of a kappa statistic

Part of an observed agreement between different observers is due to pure chance. We are interested in the agreement between observers above that of a pair of dice. The kappa statistic is the observed agreement, corrected for chance, as a fraction of the maximum agreement also corrected for chance (Svanholm, Starklint et al. 1989).

$$\kappa = \frac{\mathbf{P}\text{obs} - \mathbf{P}\text{chance}}{1 - \mathbf{P}\text{chance}}$$

2B - KAPPA SCORING OF VARIABILITY

Kappa scores were calculated for MLH1, MSH2, and p53. For each block of tissue there were 4 scores for each protein (MLH1, MSH2, p53) for both intensity and percentage score. This consisted of 2 slides scored by 2 observers i.e.

a) Slide 1 - score by MJM

b) Slide 1 - score by DM

c) Slide 2 - score by MJM

d) Slide 2 - score by DM

Each of these scores was based on a scale of 0 to 3. The weighted kappa results for these scores give a heavier weighting the greater the difference in score. The intra-slide kappa score was examined by comparing the score for (a) with the score for (c) and also the score for (b) with the score for (d). The inter-observer kappa score was examined by comparing the score for (a) with the score for (b) and also the score for (c) with the score for (d). We have included all the scores for all blocks for the calculation of the kappa scores. A guide to interpretation of kappa scores is given in table 43. The results for MLH1, MSH2 and p53 are shown in tables 24 to 26.

2B (I) - Guidelines for interpretation of kappa values

Kappa value	Degree of agreement
0.00 – 0.20	Slight
0.21 – 0.40	Fair
0.41 – 0.60	Moderate
0.61 – 0.80	Substantial
0.81 – 1.00	Almost perfect

Table 23 – Guidelines for interpretation of kappa values

A kappa result of 0.00 means any agreement may be explained by chance alone. A kappa result of 1.00 means complete agreement. A cut-off of 0.5 is often used as the lower level of an acceptable agreement corrected for chance.

2B (II) - Ki67 Kappa score

For Ki67 a different scoring system was used (see materials and methods for CAS scoring system). Only one observer generated Ki67 scores, (MJM), due to the time consuming nature of performing CAS scoring. 2 slides were again stained per block of tissue and scored on different occasions (n=59). The intra-class correlation coefficient was calculated at 0.789 (standard error = 0.053) as a measure of variability and is similar to the intra-slide kappa score.

Table 24 - MSH2 Kappa scores

MSH2 **Inter-Observer**	WEIGHTED KAPPA	STANDARD ERROR	N=
Slide 1 I-DM * Slide 1 I-MJM	0.612	0.065	105
Slide 2 I-DM * Slide 2 I-MJM	0.698	0.066	104
Slide 1 %-DM * Slide 1 %-MJM	0.587	0.112	105
Slide 2 %-DM * Slide 2 %-MJM	0.755	0.067	104
Overall intensity	**0.653**	0.046	209
Overall percentage	**0.711**	0.057	209
MSH2 **Intra-slide**			
Slide 1 I-MJM * Slide 2 I-MJM	0.604	0.072	106
Slide 1 I-DM * Slide 2 I-DM	0.590	0.069	105
Slide 1 %-MJM * Slide 2 %-MJM	0.610	0.078	106
Slide 1 %-DM * Slide 2 %-DM	0.603	0.085	105
Overall intensity	**0.602**	0.050	211
Overall percentage	**0.607**	0.057	211
MSH2 **Intra-observer**			
Overall intensity	**0.733**	0.072	46
Overall percentage	**0.692**	0.087	46

Table 25 - MLH1 Kappa scores

MLH1 **Inter-Observer**	WEIGHTED KAPPA	STANDARD ERROR	N=
Slide 1 I-DM * Slide 1 I-MJM	0.731	0.043	104
Slide 2 I-DM * Slide 2 I-MJM	0.525	0.08	106
Slide 1 %-DM * Slide 1 %-MJM	0.688	0.065	104
Slide 2 %-DM * Slide 2 %-MJM	0.524	0.087	106
Overall intensity	**0.685**	0.038	210
Overall percentage	**0.629**	0.053	210
MLH1 **Intra-slide**			
Slide 1 I-MJM * Slide 2 I-MJM	0.606	0.066	106
Slide 1 I-DM * Slide 2 I-DM	0.557	0.071	104
Slide 1 %-MJM * Slide 2 %-MJM	0.648	0.069	106
Slide 1 %-DM * Slide 2 %-DM	0.645	0.070	104
Overall intensity	**0.583**	0.048	210
Overall percentage	**0.647**	0.049	210
MLH1 **Intra-observer**			
Overall intensity	0.689	0.096	46
Overall percentage	0.646	0.099	46

Table 26 - p53 Kappa scores

P53 **Inter-Observer**	WEIGHTED KAPPA	STANDARD ERROR	N=
Slide 1 I-DM * Slide 1 I-MJM	0.800	0.042	106
Slide 2 I-DM * Slide 2 I-MJM	0.820	0.036	105
Slide 1 %-DM * Slide 1 %-MJM	0.762	0.058	106
Slide 2 %-DM * Slide 2 %-MJM	0.780	0.048	105
Overall intensity	**0.812**	0.027	211
Overall percentage	**0.773**	0.037	211
p53 **Intra-slide**			
Slide 1 I-MJM * Slide 2 I-MJM	0.902	0.019	105
Slide 1 I-DM * Slide 2 I-DM	0.910	0.024	107
Slide 1 %-MJM * Slide 2 %-MJM	0.735	0.067	105
Slide 1 %-DM * Slide 2 %-DM	0.739	0.055	107
Overall intensity	**0.905**	0.015	212
Overall percentage	**0.737**	0.04378	212
p53 **Intra-observer**			
Overall intensity	**0.851**	0.044	46
Overall percentage	**0.818**	0.080	46

2B (III) - Conclusions from kappa scores

Overall results are shown in table 27. The p53 scores were consistently the most reproducible. There was no significant difference between DM and MJM for intra-slide variability. The only kappa score less than 0.6 was for intra-slide variation of MLH1 intensity (0.583). This suggests that most variation was seen for staining technique for MLH1. However the overall intra-slide kappa scores compare favourably to a small study of 22 endometrial tissue biopsies stained for oestrogen (ER) and progesterone receptors (PgR) on two separate slides on separate occasions. Ravn et al. showed an intra-slide kappa score for either intensity (scale 0 to 3) or percentage (0 to 3) of 0.695 for ER and 0.600 for PgR (Ravn, Rasmussen et al. 1993). For intra-slide variation the p53 intensity score was significantly more reproducible than any other score with a kappa value of 0.905 ($p<0.05$ compared to p53 percentage kappa score). For both intra-observer and inter-observer kappa scores there was no significant difference seen between the percentage and intensity scores in reproducibility. The intra and inter-observer kappa scores compare very favourably to a detailed study of variation between 3 observers over time by Adams et al. (Adams, Green et al. 1999). For p53 and EGFR, using a 0-3 scale for intensity scoring, they showed intra- and inter-observer kappa scores of 0.32 and 0.49 respectively.

We have, therefore, shown remarkable reproducibility of the scoring system between 2 independent observers and between 2 slides of the same tissue and on 2 different occasions for MLH1, MSH2 and p53.

Table 27 - Overall Kappa values

INTER-OBSERVER	WEIGHTED KAPPA	STANDARD ERROR	N=
MSH2 Intensity	0.653	0.046	209
MSH2 Percentage	0.711	0.057	209
MLH1 Intensity	0.685	0.038	210
MLH1 Percentage	0.629	0.053	210
P53 Intensity	0.812	0.027	211
P53 Percentage	0.773	0.037	211

INTRA-SLIDE	WEIGHTED KAPPA	STANDARD ERROR	N=
MSH2 Intensity	0.602	0.050	211
MSH2 Percentage	0.607	0.057	211
MLH1 Intensity	0.583	0.048	210
MLH1 Percentage	0.647	0.049	210
P53 Intensity	0.905	0.015	212
P53 Percentage	0.737	0.043	212
Ki67 Percentage	0.789	0.053	59

INTRA-OBSERVER	WEIGHTED KAPPA	STANDARD ERROR	N=
MSH2 Intensity	0.733	0.072	46
MSH2 Percentage	0.692	0.087	46
MLH1 Intensity	0.689	0.096	46
MLH1 Percentage	0.646	0.099	46
P53 Intensity	0.851	0.044	46
P53 Percentage	0.818	0.080	46

2C - PERCENTAGE SCORING OF VARIABILITY

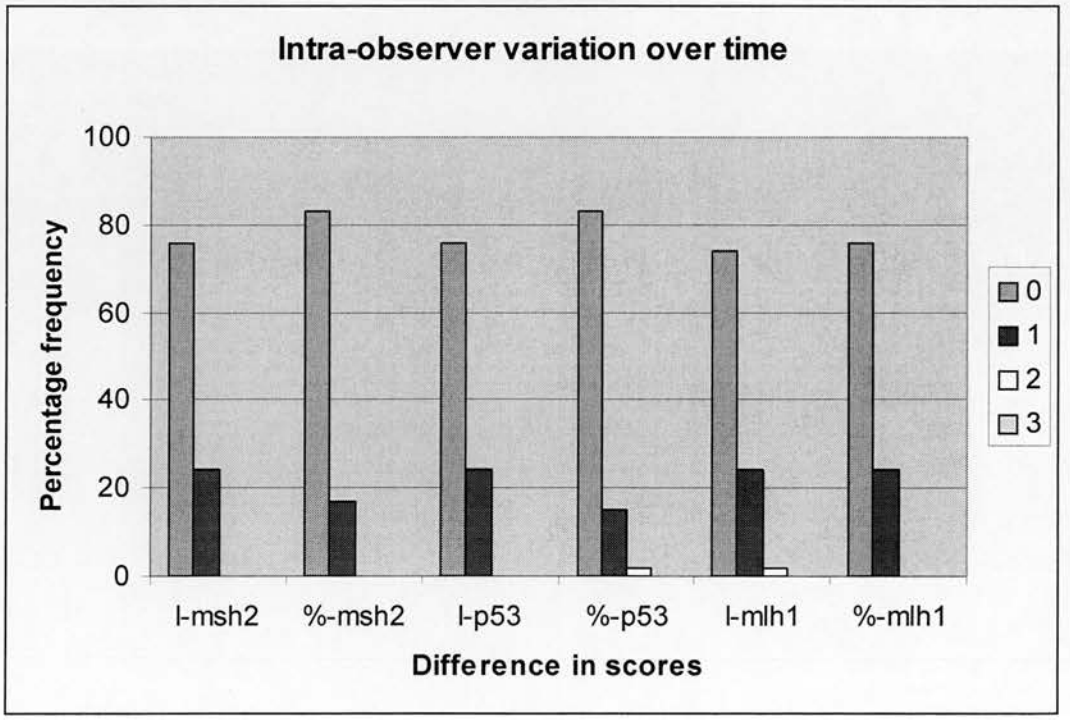
This method examines the difference between the 2 scores and the number of times the scores vary by these differences. As our scoring scale is from 0 to 3 the difference between the scores can vary from a minimum of 0 to a maximum of 3. This is then expressed as a percentage of the total number of slides scored.

2C (I) - Intra-observer variation over time

The results are shown in the table 28 and figure 22. The scores were the same for the same slide scored for a second time after 6 months in over 70% of the 46 slides examined. This compares favourably with a study of intra-observer variation in ER scoring using a similar 0-3 scale for both intensity and percentage staining. On 50 breast cancer specimens Remmele et al. showed an agreement of 78% for intensity and 86% for percentage score (Remmele, Hildebrand et al. 1986). In the current study there was no significant difference between the three antibodies examined. On only 2 occasions out of a possible 276 (6x46) did the score vary by 2 and never by 3.

Table 28 – Intra-observer variation over time

INTRA-OBSERVER VARIATION OVER TIME (N=46)						
Difference in scores	I-MSH2	%-MSH2	I-p53	%-p53	I-MLH1	%-MLH1
0	35 76%	38 83%	35 76%	38 83%	34 74%	35 76%
1	11 24%	8 17%	11 24%	7 15%	11 24%	11 24%
2	0 0%	0 0%	0 0%	1 2%	1 2%	0 0%
3	0 0%	0 0%	0 0%	0 0%	0 0%	0 0%

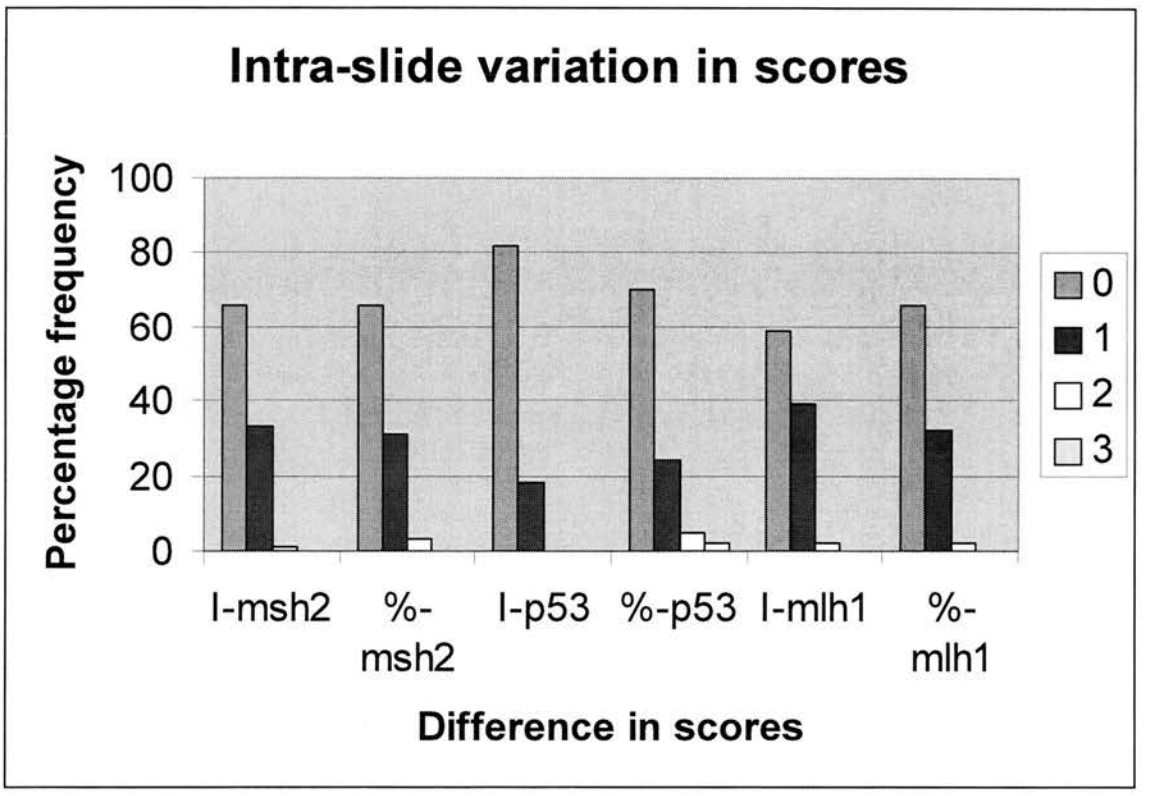


2C (II) - Intra-slide variation

Results are shown in the table 29 and figure 23 below. p53 scores were more consistently in agreement than those for MSH2 and MLH1 with 70% and 82% of scores the same for percentage and intensity of p53 respectively. The worst concordance was for intensity of MLH1 with only 59% of scores remaining the same. There were very few scores varying by 2 or 3. Interestingly a significant number of these were for percentage score of p53 suggesting when there is a variation in p53 %-score this tends to be of a larger scale.

Table 29 – Intra-slide variation

INTRA-SLIDE VARIATION (N= MAX. 212)						
Difference in scores	I-MSH2	%-MSH2	I-p53	%-p53	I-MLH1	%-MLH1
0	138 66%	138 66%	174 82%	148 70%	124 59%	140 66%
1	70 33%	66 31%	38 18%	51 24%	83 39%	67 32%
2	3 1%	7 3%	0 0%	11 5%	5 2%	5 2%
3	0 0%	0 0%	0 0%	2 1%	0 0%	0 0%

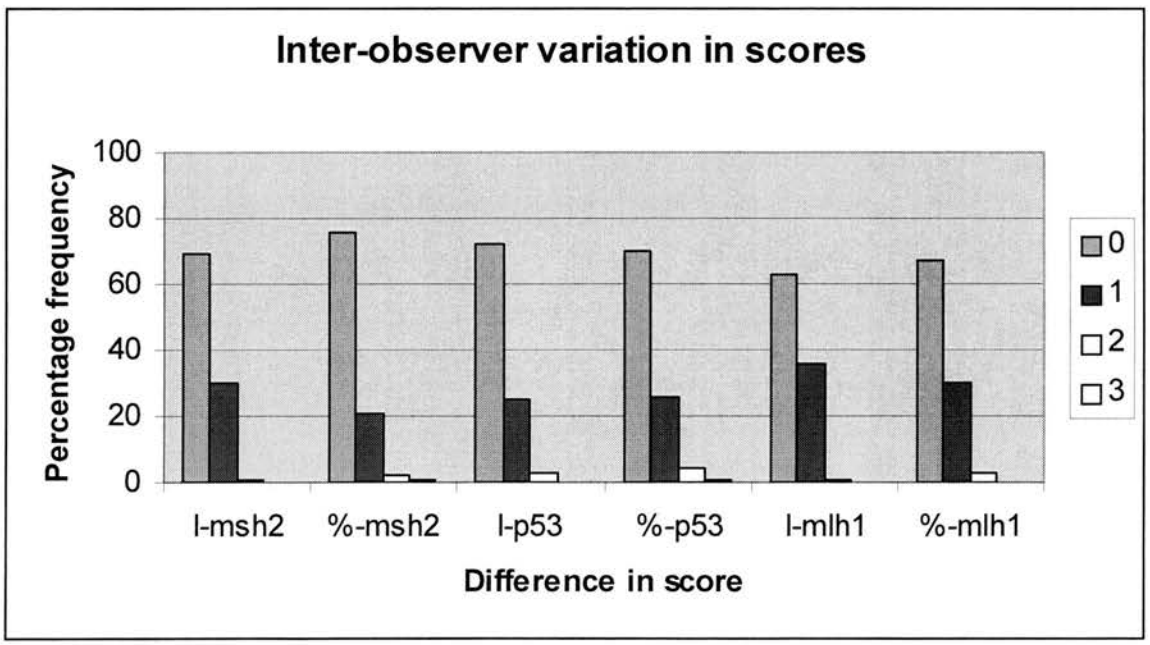


2C (III) - Inter-observer variation

The results are shown in the table 30 and figure 24. The overall agreement in scores is slightly lower at around two thirds for all the antibodies. The MLH1 scores show slightly more variation than the other antibodies. Again the number of variations of 2 or 3 are very small. These compare favourably to the results by Remmele et al 1986 for ER immunohistochemistry on a similar 0-3 scale (Remmele, Hildebrand et al. 1986). Between experienced observers they showed absolute agreement in 58 and 60% for intensity and 78 and 82% for percentage scores.

Table 30 – Inter-observer variation in scores

INTER-OBSERVER VARIATION (N= MAX. 211)						
Difference in scores	I-MSH2	%-MSH2	I-p53	%-p53	I-MLH1	%-MLH1
0	145 69%	159 76%	153 72%	147 70%	131 63%	139 67%
1	62 30%	44 21%	52 25%	54 25.5%	74 36%	62 30%
2	2 1%	4 2%	6 3%	9 4%	3 1%	7 3%
3	0 0%	2 1%	0 0%	1 0.5%	0 0%	0 0%



2D - VARIABILITY OF IMMUNOSCORES - CONCLUSIONS

- This is the first study to examine reliability of scoring for MLH1 and MSH2 immunohistochemistry in any detail and shows remarkable reproducibility of the scoring system between 2 independent observers and between 2 slides of the same tissue and on 2 different occasions.

- Only intensity of MLH1 score fell below a kappa score of 0.6 (0.583) for intra-slide variability i.e. between IHC runs on separate days.

- Scoring for p53 was found to be the most reliable between scorers, over time and over different IHC runs.

- Testing of Ki67 scoring for reliability was limited due to the time consuming nature of CAS scoring but was found to be good (0.789) between IHC runs on separate days (intra-slide).

- Percentage scoring of reliability is often quoted in the literature instead of kappa scores. Using percentage scoring of reliability we confirmed our above findings and these compared favourably to other IHC scoring used routinely.

Chapter 6 - IMMUNOHISTOCHEMISTRY

A – RESULTS OF IMMUNOSCORES AND EXAMPLES

A – (i) PICTURES OF IMMUNOHISTOCHEMISTRY

Pictures of typical immunohistochemistry are shown in the following pages. These are shown at x50 magnification. The index for the pictures is as follows;

Picture 1 – MLH1 staining score 3+3 ovarian tumour

Picture 2 – MLH1 staining score 2+2, ovarian tumour

Picture 3 – MLH1 staining score 1+1, ovarian tumour

Picture 4 – MLH1 staining score 3+3, ovarian tumour

Picture 5 – MSH2 staining score 1+1, ovarian tumour

Picture 6 – MSH2 staining score 2+2, ovarian tumour

Picture 7 – MSH2 staining score 3+3, ovarian tumour

Picture 8 – p53 staining score 1+1, ovarian tumour

Picture 9 – p53 staining score 2+2, ovarian tumour

Picture 10 – p53 staining score 3+3, ovarian tumour

Picture 11 – Ki67 staining, low, ovarian tumour

Picture 12 – Ki67 staining, high, ovarian tumour

Picture 13 – Control slide, ovarian cell line A2780, +ve for MLH1

Picture 14 – Control cell line A2780, +ve for MSH2

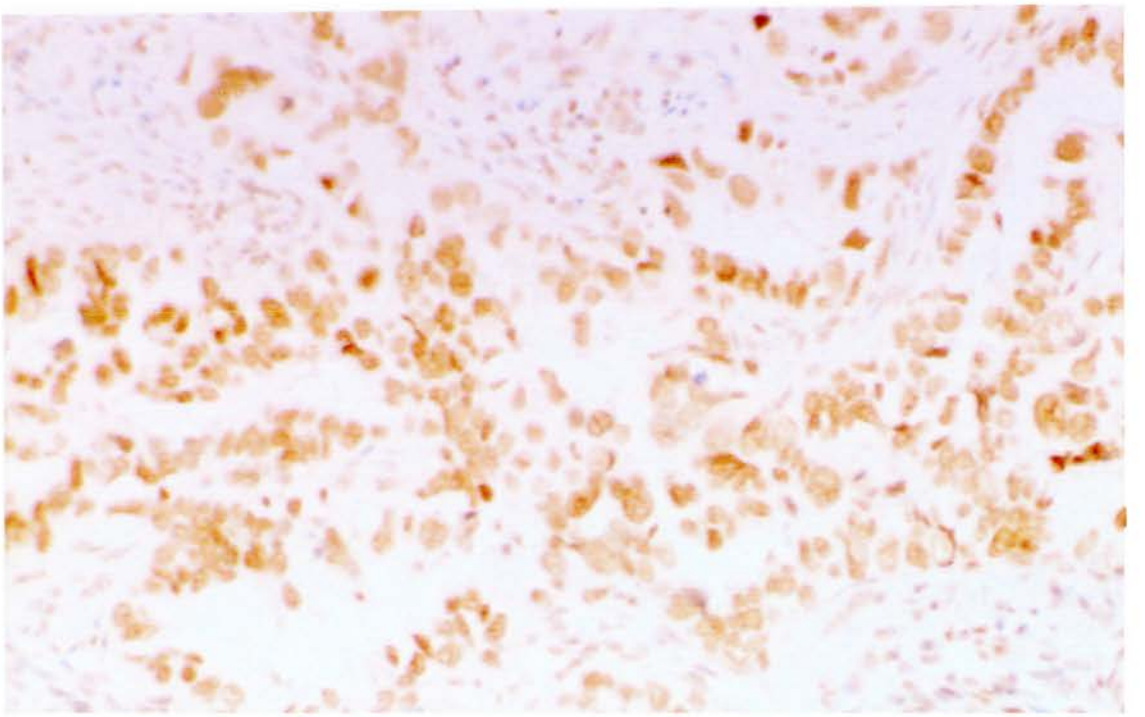
Picture 15 – Control slide, ovarian cell line CP70, -ve for MLH1

Picture 16 – Control slide, colon cancer cell line LOVO 1, -ve for MSH2

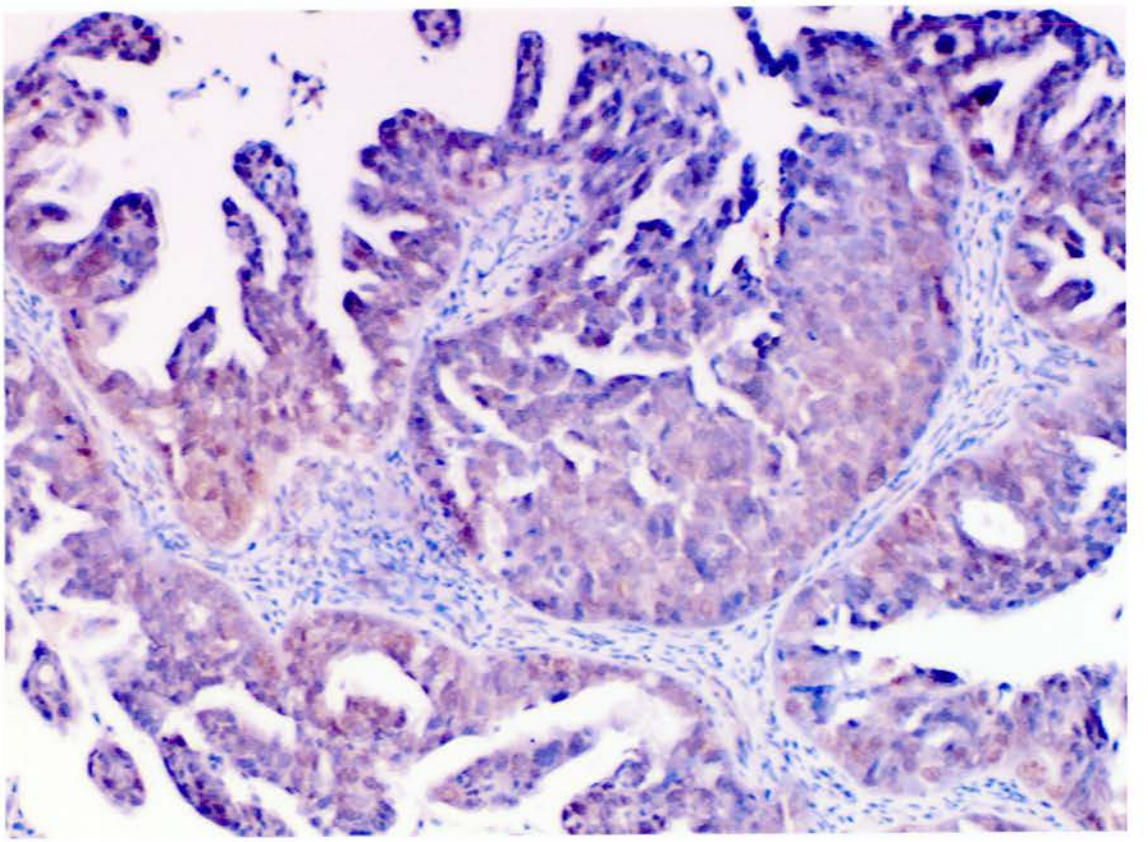
Picture 17 – Normal colon, Crypts of Lieberkuhn, +ve for MLH1

Picture 18 – Normal colon, Crypts of Lieberkuhn, +ve for Ki67

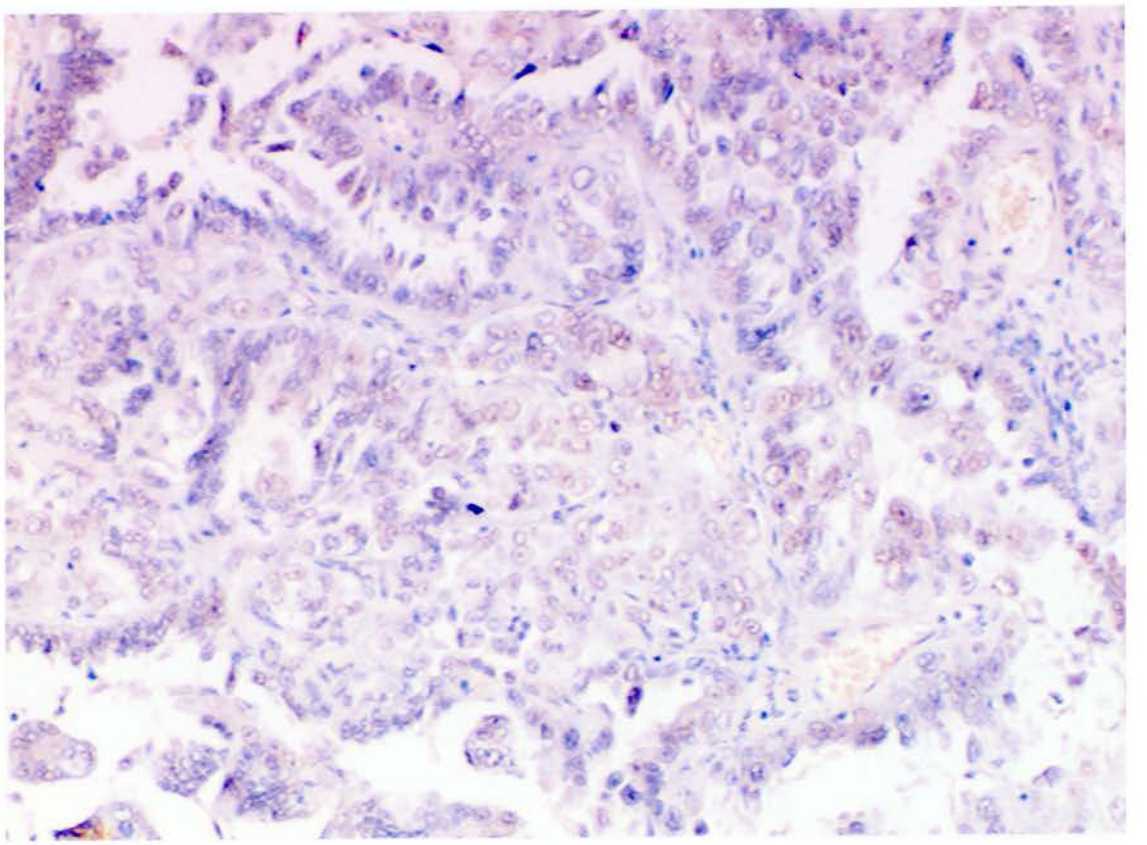
Picture 1 – MLH1 staining score 3+3 ovarian tumour



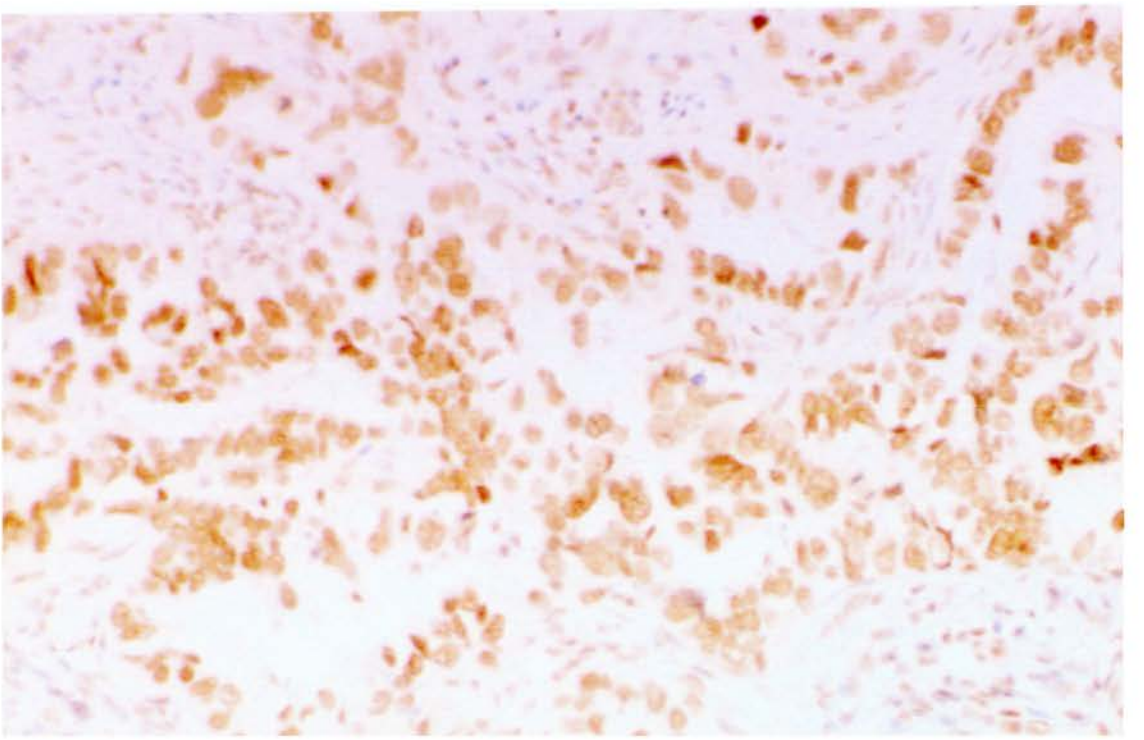
Picture 2 – MLH1 staining score 2+2, ovarian tumour



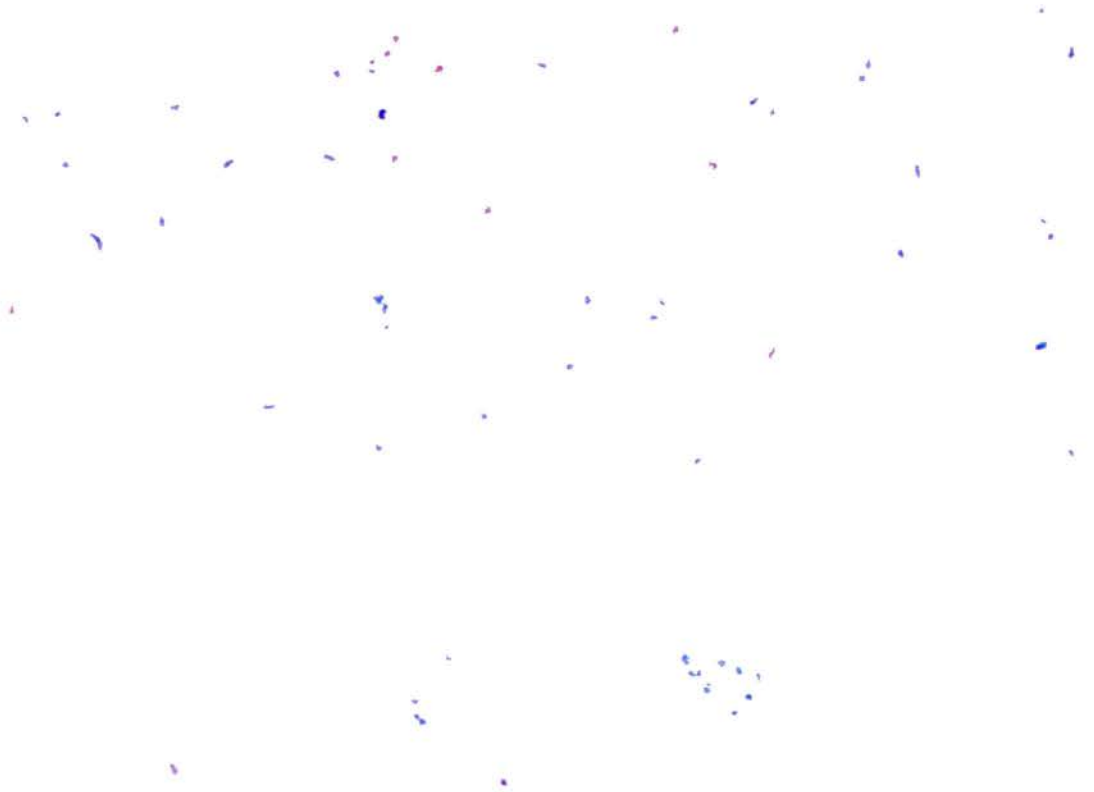
Picture 3 – MLH1 staining score 1+1, ovarian tumour



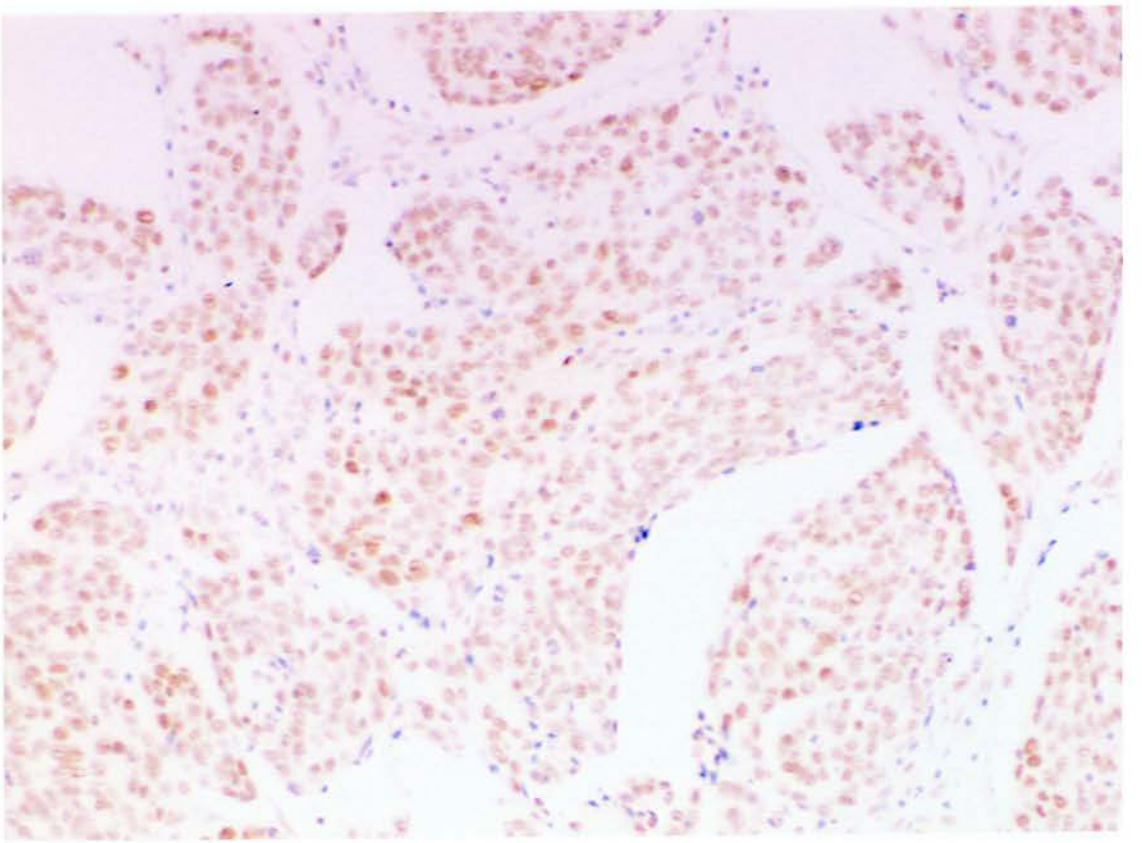
Picture 4 – MLH1 staining score 3+3, ovarian tumour



Picture 5 – MSH2 staining score 1+1, ovarian tumour

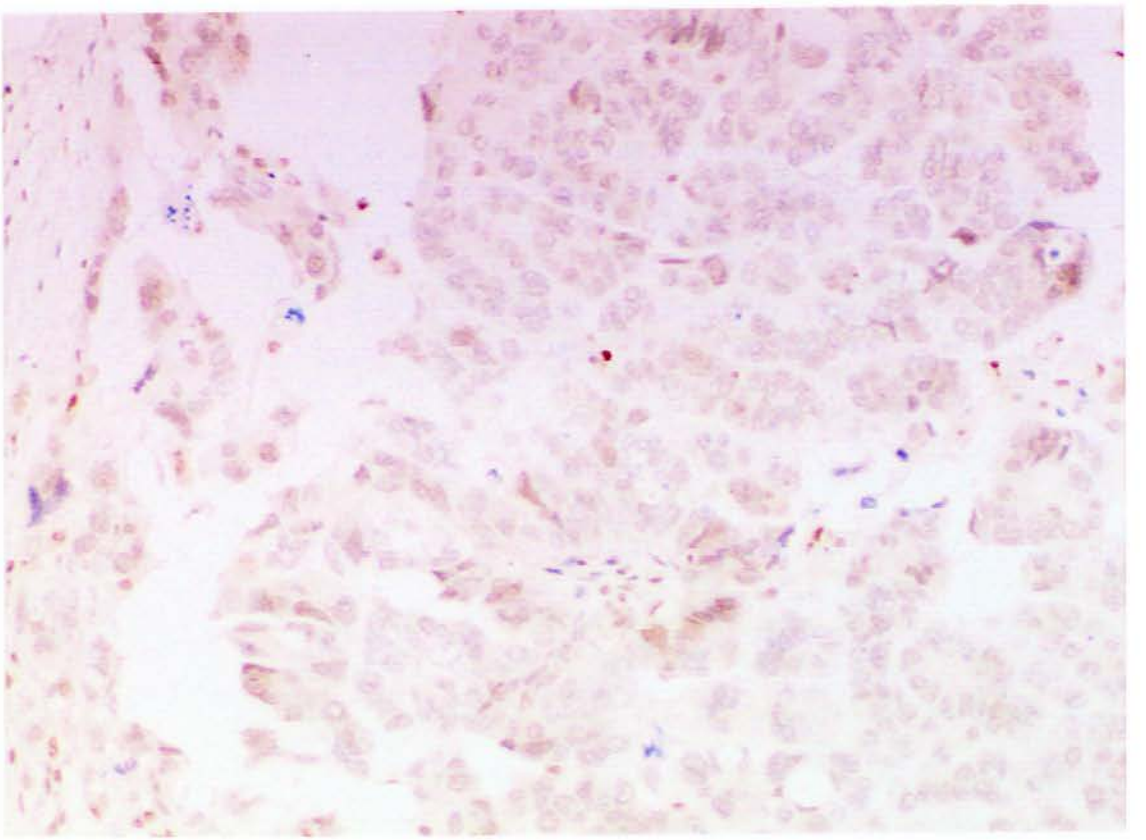


Picture 6 – MSH2 staining score 2+2, ovarian tumour



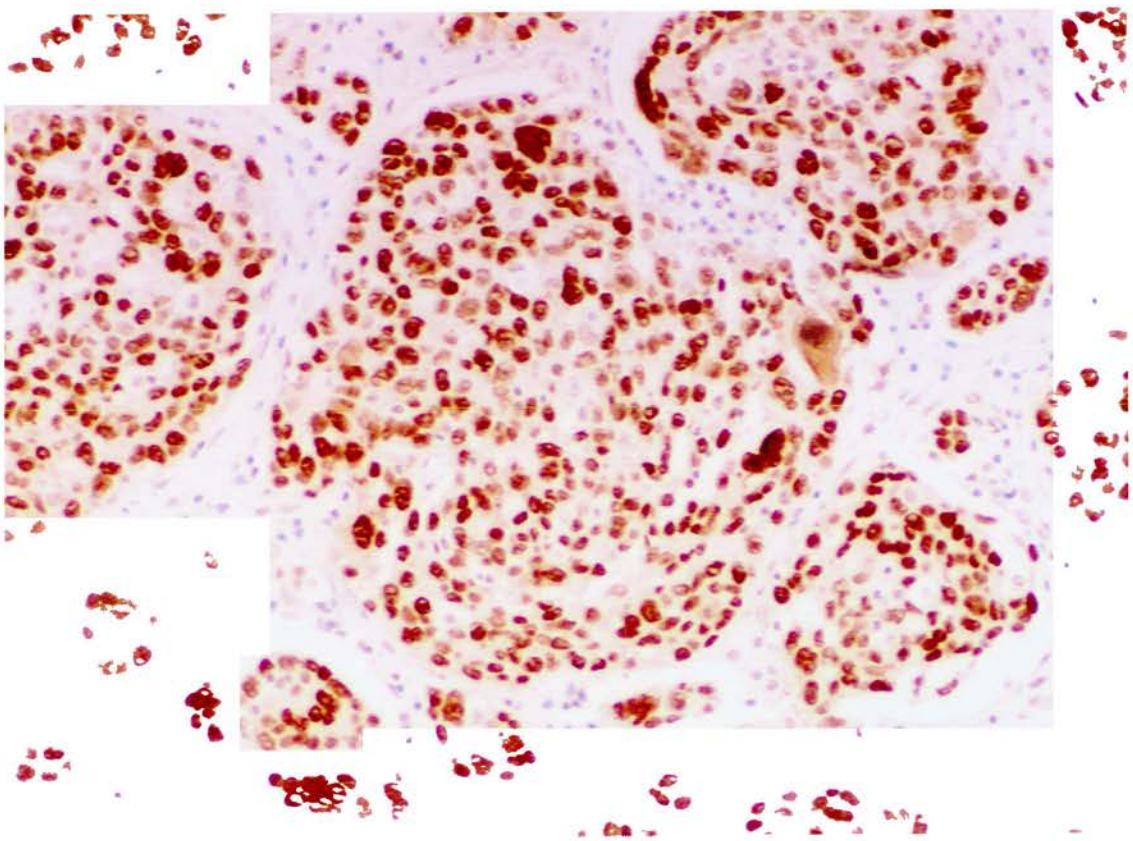
Picture 7 – MSH2 staining score 3+3, ovarian tumour

Picture 8 – p53 staining score 1+1, ovarian tumour

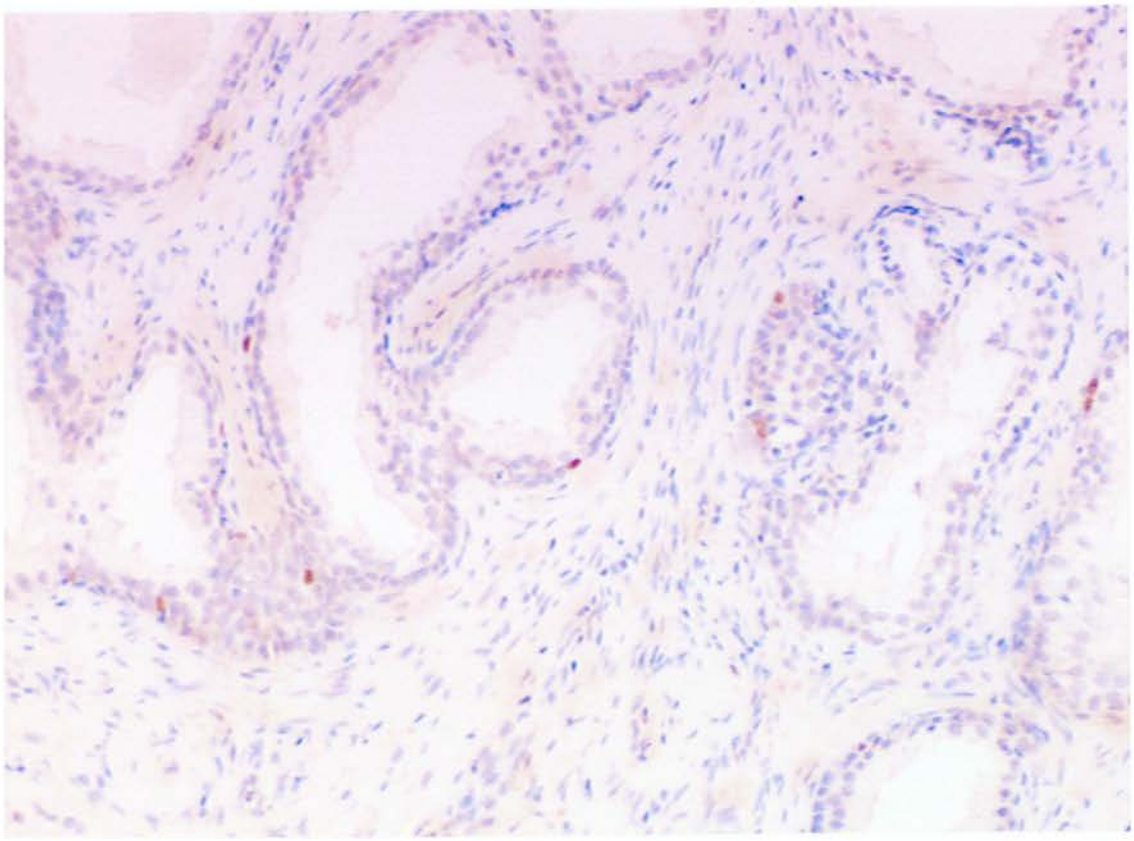


Picture 9 – p53 staining score 2+2, ovarian tumour

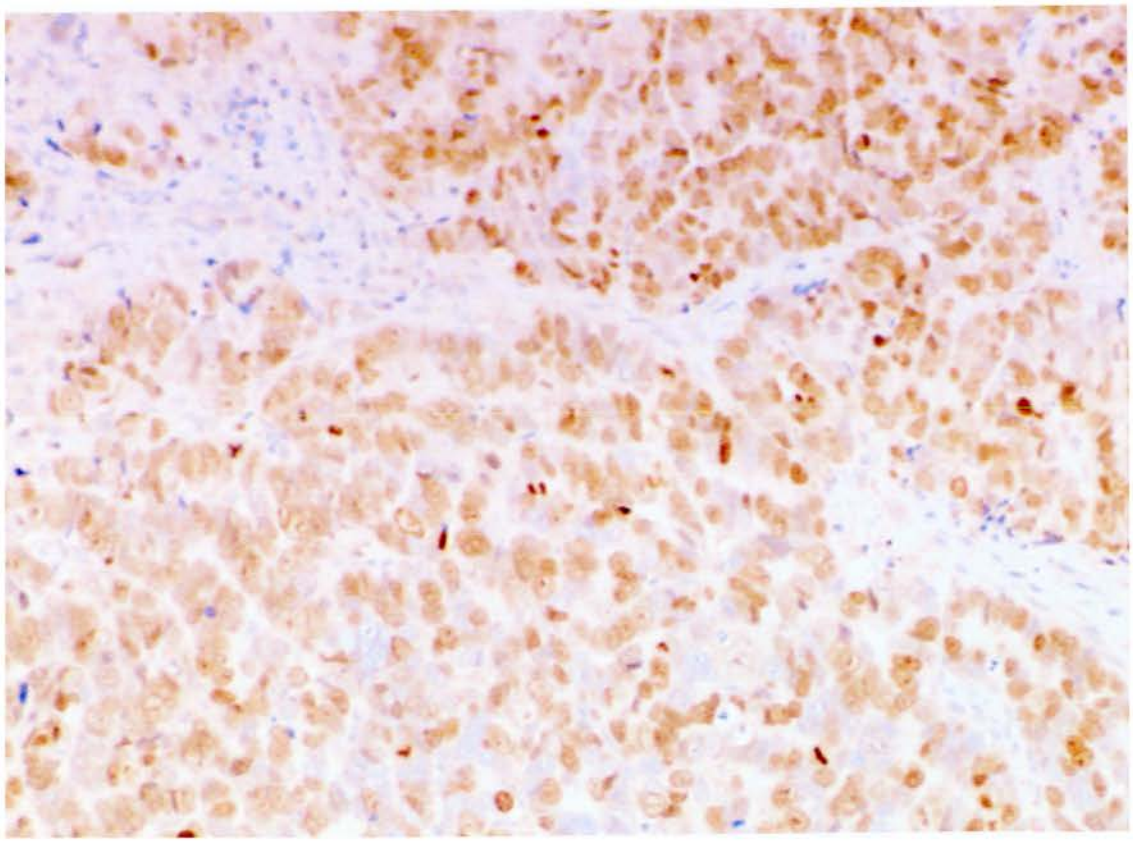
Picture 10 – p53 staining score 3+3, ovarian tumour



Picture 11 – Ki67 staining, low, ovarian tumour

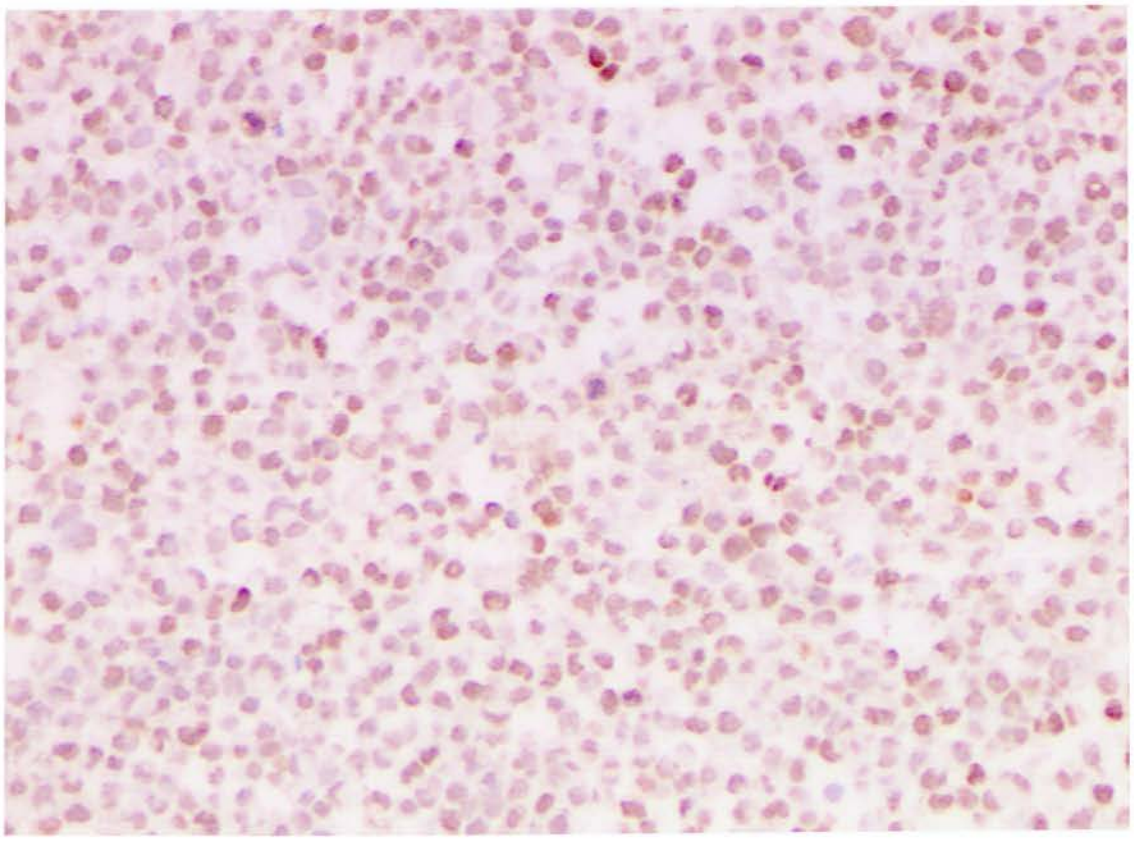


Picture 12 – Ki67 staining, high, ovarian tumour

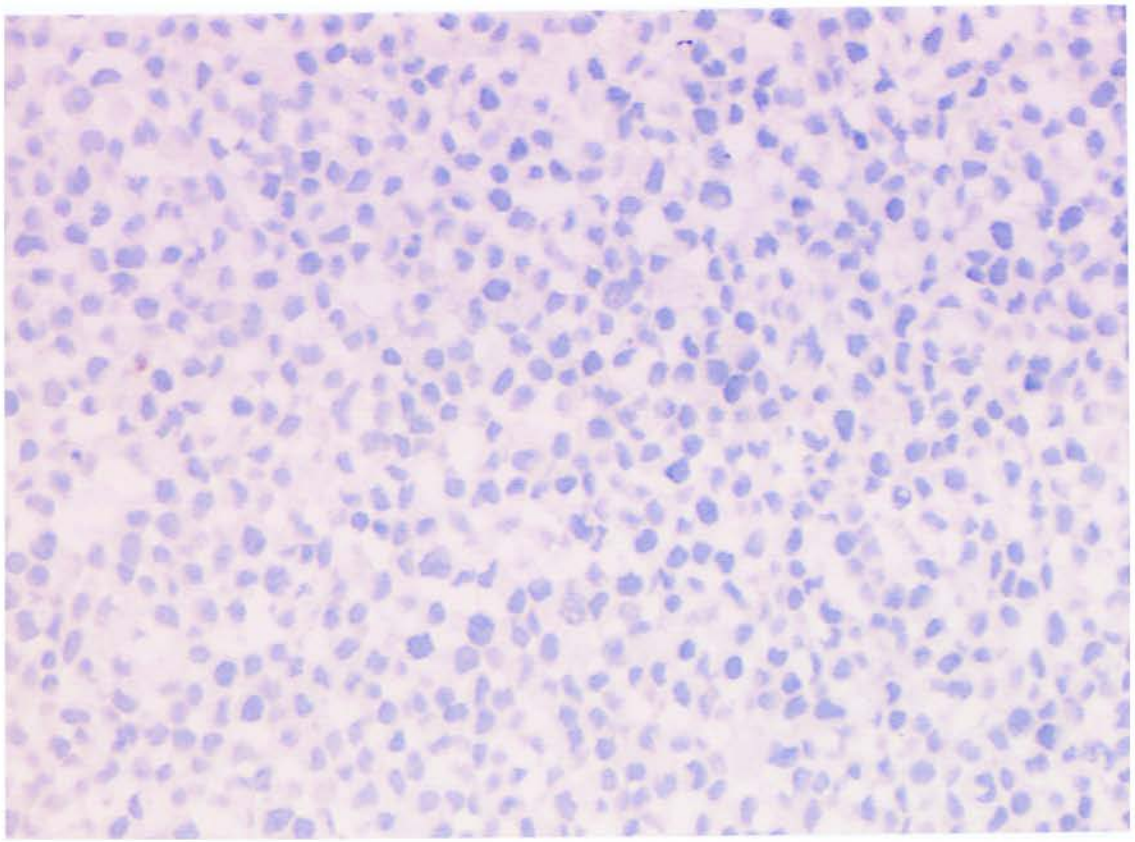


Picture 13 – Control slide, ovarian cell line A2780, +ve for MLH1

Picture 14 – Control cell line A2780, +ve for MSH2

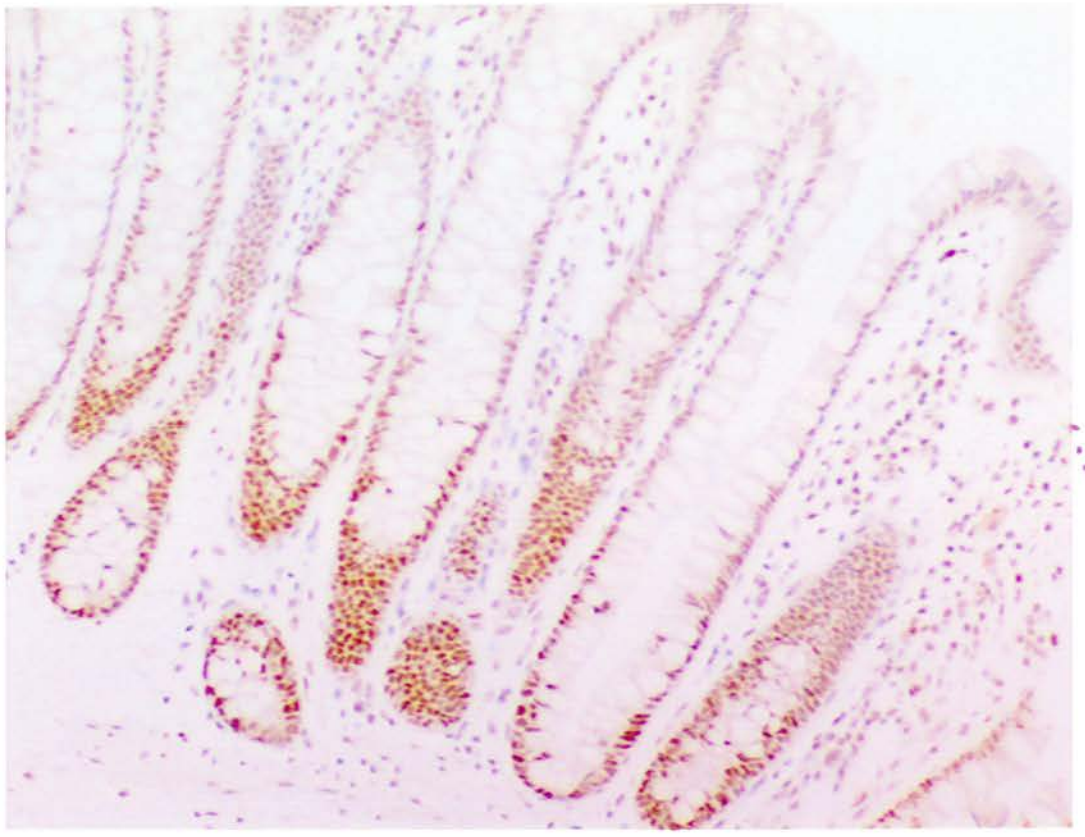


Picture 15 – Control slide, ovarian cell line CP70, -ve for MLH1

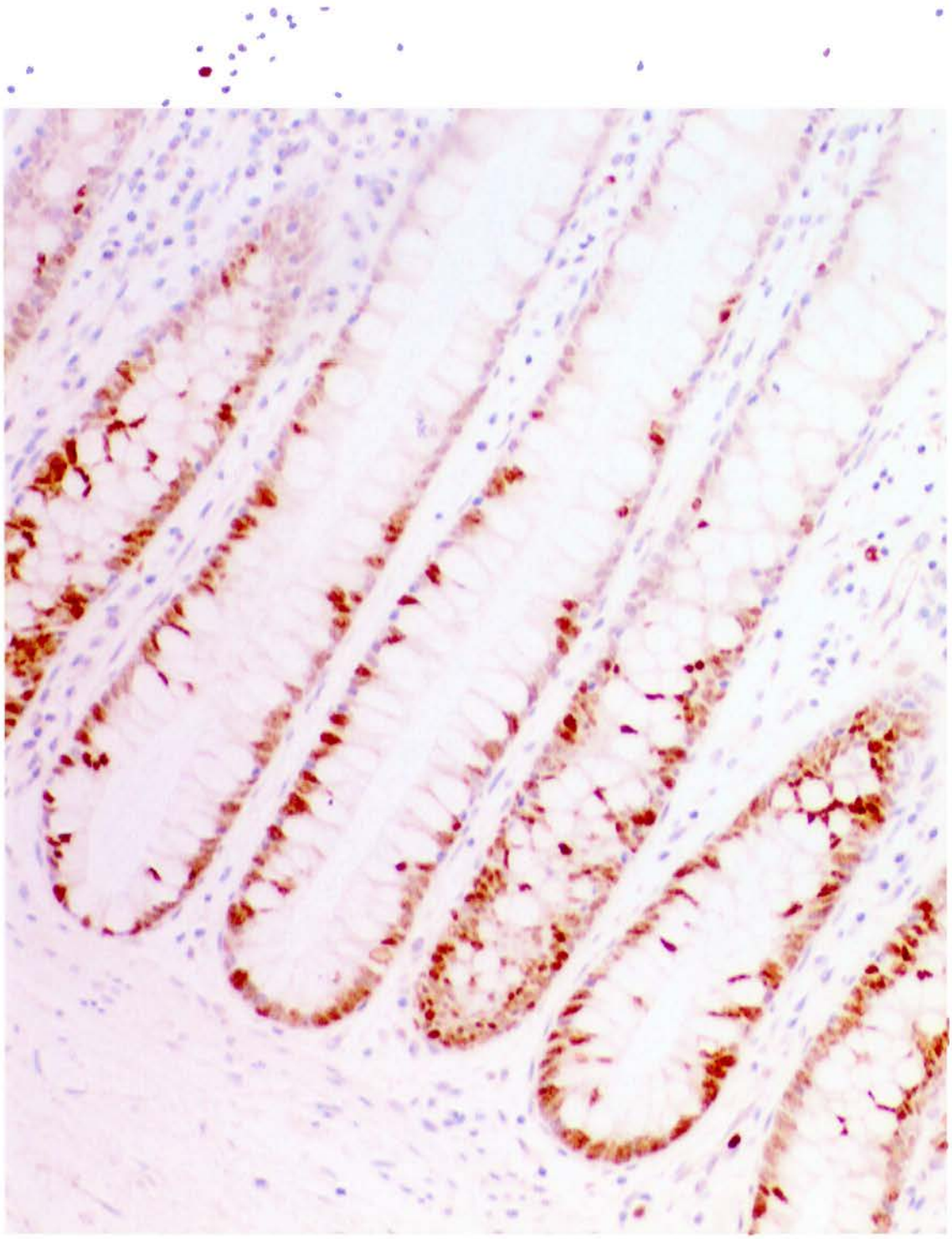


Picture 16 – Control slide, colon cancer cell line LOVO 1, -ve for MSH2

Picture 17 – Normal colon, Crypts of Lieberkuhn, +ve for MLH1



Picture 18 – Normal colon, Crypts of Lieberkuhn, +ve for Ki67



A – (ii) DISTRIBUTION OF IMMUNOSCORES (N=97)

The statistics of the immunoscores are shown in the table 31. Scores were not normally distributed with an excess of high scores for I-MSH2, %-p53, %-MLH1 and %-MSH2. This is shown in the histograms (figure 2, 3 and 4). This means for the intensity scores only about one third of scores were below the middle value of 1.5 (21%, 32% and 34% for MSH2, MLH1 and p53 respectively). For percentage scores this was more striking with 12%, 14% and 26% below the middle value of 1.5 for MSH2, MLH1 and p53 respectively.

Table 31 - Statistics of all immunoscores (n=97)

	I-MSH2	%-MSH2	%-KI67	I-P53	%-P53	I-MLH1	%-MLH1
Count	97	97	95	97	97	97	97
Mean	2.007	2.354	16.403	2.075	2.178	1.888	2.269
Median	2	2.5	15	2.5	2.625	2	2.5
SD	0.6034	0.7302	11.5281	0.9828	0.9859	0.6544	0.6609
95%CI	0.1201	0.1453	2.3181	0.1955	0.1962	0.1302	0.1315
1st quartile	1.75	2	6.25	1	1.75	1.5	2
3rd quartile	2.5	3	24	3	3	2.375	2.75
Min.	0	0	0.5	0	0	0	0
Max.	3	3	47	3	3	3	3

Figure 2 – Histogram of % Ki67 scores, N=95. This shows a skewed distribution weighted to the lower scores with a tail of higher scores.

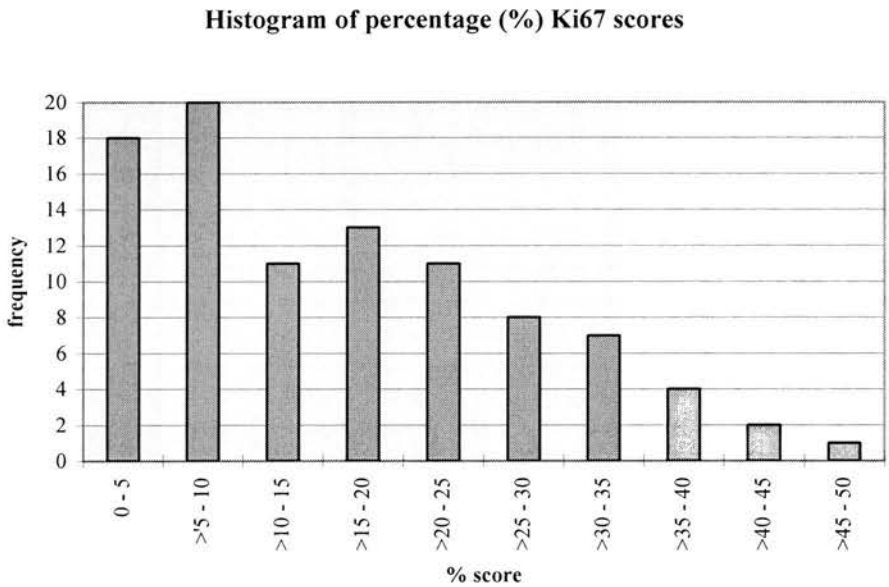


Figure 3 – Histogram of % scores for MLH1, p53 and MSH2, N=97. This shows a skewed distribution of higher scores.

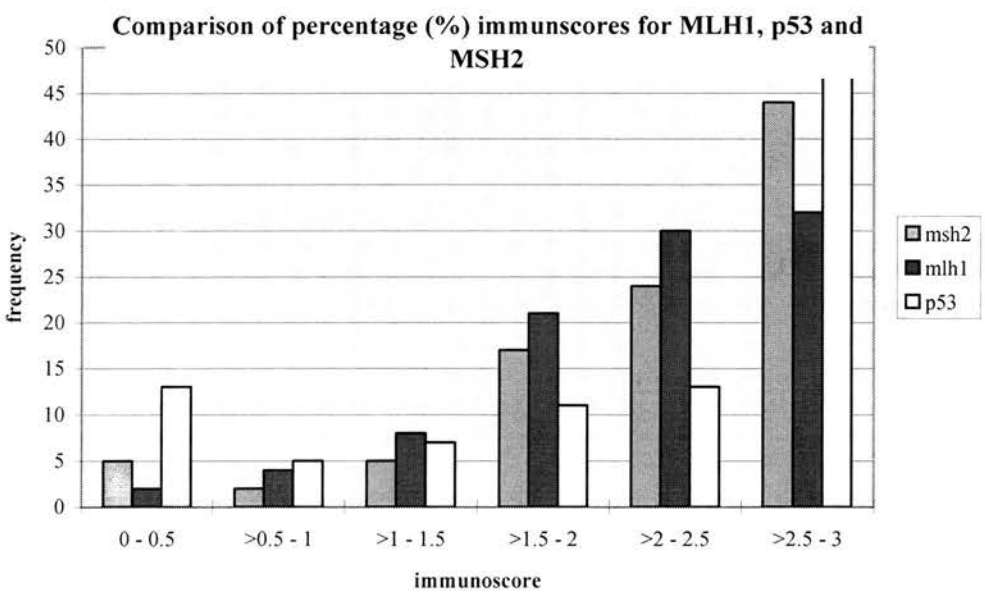
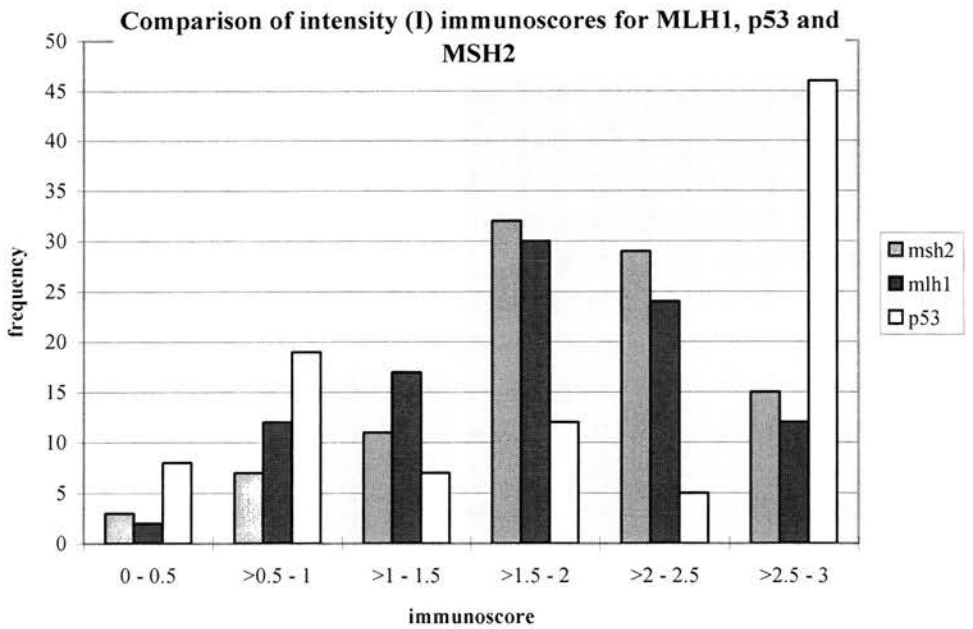


Figure 4 – Histogram of intensity scores for MLH1, p53 and MSH2, N=97. This shows a skewed distribution of higher scores.



A (iii) – COMPARISON WITH OTHER STUDIES

We were expecting the number of negative (i.e. 0 stain) cases for mismatch repair proteins to be of the order of 10 to 17% from the literature on microsatellite analysis (see introduction). Interesingly we only found 7 out of 97 blocks to be totally negative for MLH1 and/or MSH2 (7.2%). There has only been one other study of immunohistochemistry for DNA mismatch repair proteins in ovarian cancer (Samimi, Fink et al. 2000). They do not quote the statistics of their immunoscores but from the histogram of the results (figure 2 in the paper) it appears they found similar results. A comparison of the percentage scores between the 2 studies is shon in table 32 below.

Table 32 – Comparison of the distribution of percentage staining scores between this study and Samimi study.

PERCENTAGE SCORE	0	1	2	3
This study MLH1 (n=97)	2.1%	4.1%	29.9%	63.9%
Samimi study MLH1 (n=62)	3.3%	6.6%	39.3%	50.8%
This study MSH2	5.2%	2.1%	22.7%	70.1%
Samimi study MSH2	1.6%	3.2%	32.3%	62.9%

B – HETEROGENEITY OF STAINING BETWEEN AND WITHIN TISSUES i.e. INTRABLOCK VARIATION (RESULTS FROM REPEAT BLOCKS) (N=13)

Tissue removed at operation and sent to the pathology laboratory is often from more than one organ e.g. ovary, omentum, uterus. For each of these organs multiple blocks are cut from representative areas e.g. tumour, normal looking tissue, resection margins. Therefore from any one operation multiple blocks are obtained from different tissues and within the same tissue. These are embedded in paraffin for later sectioning and examination under light microscopy. Blocks consist of approximately $1cm^3$ of tissue.

In this study we managed to obtain more than one block from the same operation in only 13 patients. In 6 patients the 2 blocks were from different tissue. In the remaining 7 the blocks were taken from the same tissue. These pairs were scored as before and the scorers were unaware of the pairing of various slides. Despite the small number we were interested to examine the variability of the scores given for these 13 pairs. Results are shown in table 33. The variability consists of the sum from; (1) variability of staining; (2) variability of scoring; (3) variability of protein expression within the same tissue (n=7) and (4) variability of protein expression from different tissues (n=6). The variability from (1) and (2) is examined and described under the kappa scores (results section 2). Therefore these results can be used to give some idea of the variability due to different protein expression in and within tissues. The numbers are too small to perform kappa scoring but we saw a surprisingly small

mean difference of less than 0.5 for MSH2, MLH1 and p53 expression. The difference with Ki67 was more striking with a mean difference of 6.46 and range of 1 to 17 difference in %-scores. On paired 2-tailed t-test the difference in I-MSH2 was found to reach significance (p=0.017). However, this significance was lost if same tissue or different tissues were considered. The individual results for the 13 patients are shown in figures 5-7. The results suggest that there is some but not a significant amount of variability between and within tissues of the IHC staining. The small number of samples examined may have missed smaller amounts of variability but our results suggest this is less than 0.5 of a score. For the purpose of the main study the mean of the two scores was taken.

Table 33 - Statistics of the difference in scores between 2 blocks taken at the same time from the same patient (n=13)

	%-KI67	I-MSH2	%-MSH2	I-MLH1	%-MLH1	I-P53	%-P53
Mean	6.46	0.37	0.42	0.38	0.37	0.19	0.35
Paired 2-tailed t-test (p value)	0.86	0.017	0.15	1.00	0.5	0.087	0.19
Range	1-17	0-1	0-1.25	0-1.75	0-1	0-0.75	0-1.75

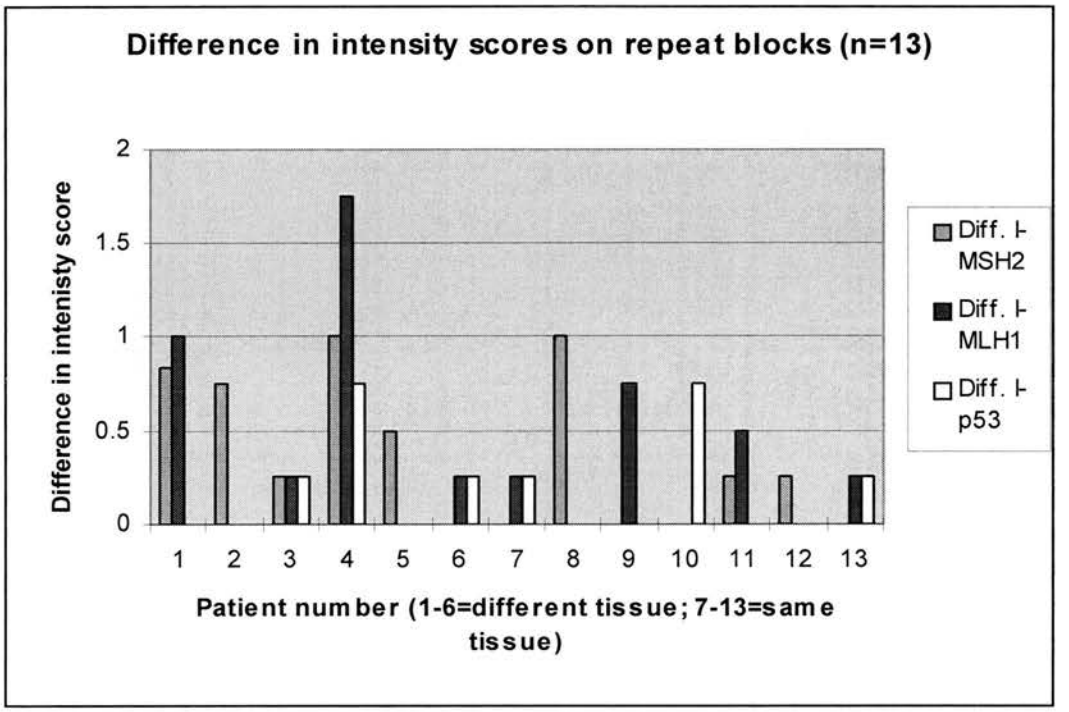


Figure 5 - Difference in intensity scores on repeat blocks (n=13)

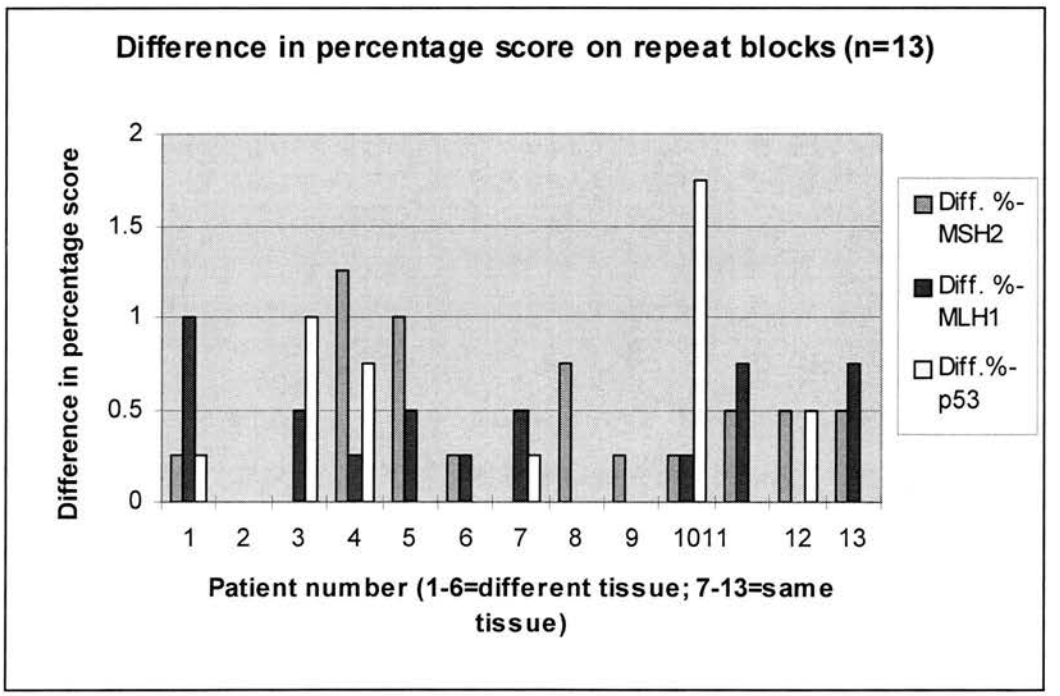
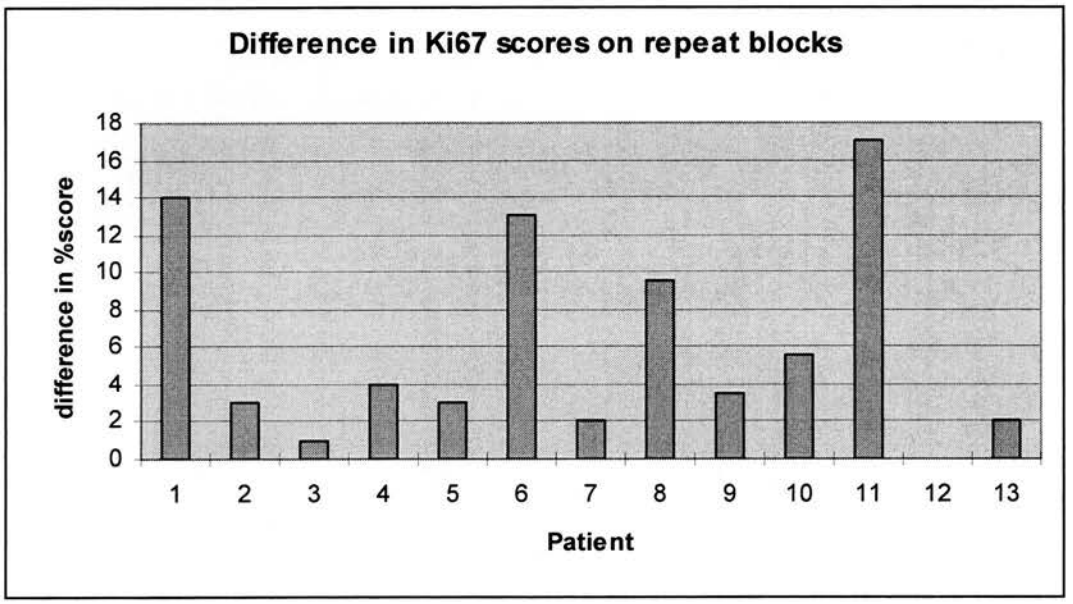


Figure 6 – Difference in percentage score on repeat blocks

Figure 7 – Difference in % Ki67 scores on repeat blocks



C – CORRELATION BETWEEN IMMUNOSCORES

The concept of scoring for both intensity of stain (I-score) and percentage of cells positive (%-score) has been accepted over the years. We examined the correlation between the % and I-scores by Spearman Rank correlation coefficients. We found a close correlation with % and I of p53 and also % and I of MLH1 ($r=0.88$ and 0.78; $p<0.001$ respectively). For I and % of MSH2 the correlation was less close but still significant ($r=0.50$; $p<0.001$). This is shown in figure 8.

We also found a positive association between the expression of the two MMR proteins studied with a weak positive correlation between MLH1 and MSH2 %-scores ($r=0.30$; $p=0.022$). This is shown in figure 9.

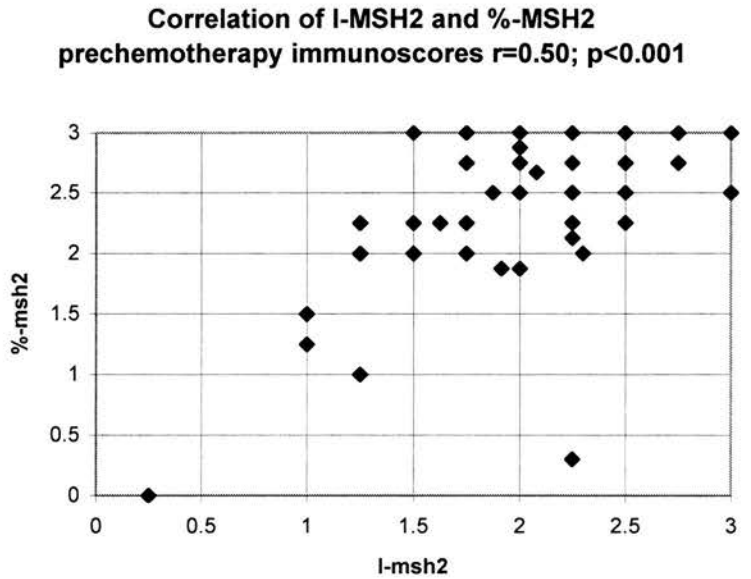
Because of the previous finding of increased expression of MMR proteins in proliferating cells (Wilson, Ewel et al. 1995; Leach, Polyak et al. 1996) the association of MMR, p53 and Ki67 pre-chemotherapy immunoscores was studied by Spearman rank correlation coefficients. The results are shown in table 34. Ki67 scores showed a positive correlation with both I- and %-score for MLH1 and MSH2 %-score ($r= 0.39$; 0.46 and 0.44; $p= 0.002$; <0.001 and 0.001 respectively) but not p53 scores. This is shown in figure 10. This confirms increasing expression of MMR proteins with increasing proliferation.

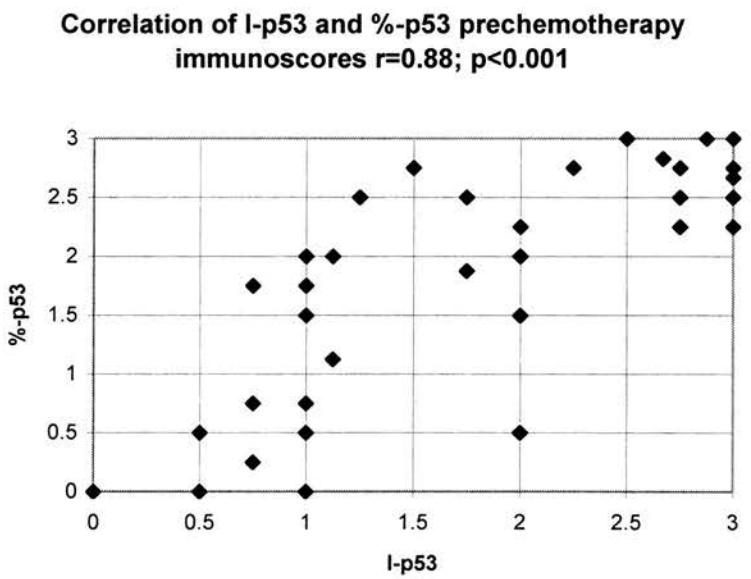
Table 34 - Spearman rank correlation coefficients between immunoscores. N=97

	I-MSH2	%-MSH2	%-KI67	I-P53	%-P53	I-MLH1	%-MLH1
I-MSH2	NA	**0.50;** ***<0.001***	0.15; *0.27*	0.06; *0.65*	0.053; *0.69*	0.24; *0.073*	0.20; *0.13*
%-MSH2	**0.50;** ***<0.001***	NA	**0.44;** ***0.001***	-0.097; *0.47*	-0.0029 *0.98*	0.17; *0.21*	**0.30;** ***0.022***
%-Ki67	0.15; *0.27*	**0.44;** ***0.001***	NA	0.012; *0.93*	0.098; *0.47*	**0.39;** ***0.002***	**0.46;** ***<0.001***
I-P53	0.06; *0.65*	-0.097; *0.47*	0.012; *0.93*	NA	**0.88;** ***<0.001***	0.20; *0.14*	0.063; *0.64*
%-P53	0.053; *0.69*	-0.0029 *0.98*	0.098; *0.47*	**0.88;** ***<0.001***	NA	0.20; *0.13*	0.14; *0.28*
I-MLH1	0.24; *0.073*	0.17; *0.21*	**0.39;** ***0.002***	0.20; *0.14*	0.20; *0.13*	NA	**0.78;** ***<0.001***
%-MLH1	0.20; *0.13*	**0.30;** ***0.022***	**0.46;** ***<0.001***	0.063; *0.64*	0.14; *0.28*	**0.78;** ***<0.001***	NA

- Spearmank Rank correlation coefficients (r) shown above
- p values shown below in italics
- Significant ($p \leq 0.05$) values shown in bold
- NA = not applicable (scores are derived from each other)

Figure 8 - Correlation of intensity and percentage scores





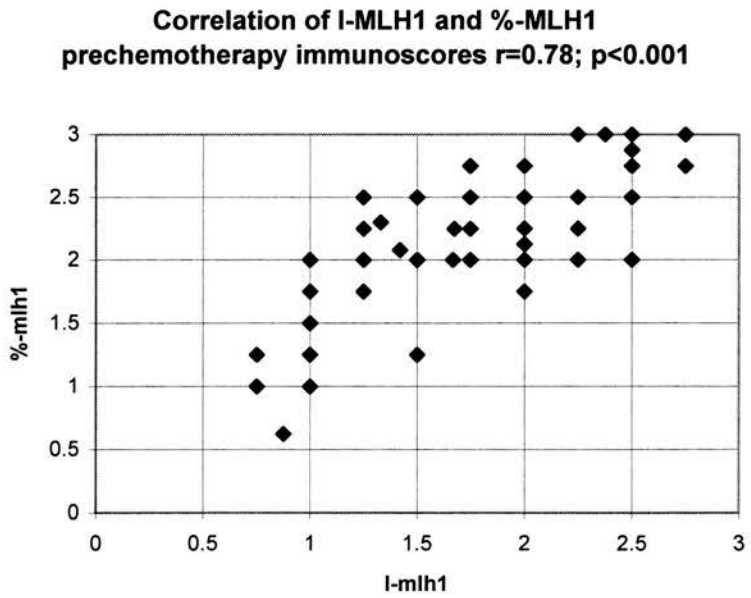

Correlation of I-MLH1 and %-MLH1
prechemotherapy immunoscores r=0.78; p<0.001
%-mlh1
I-mlh1
0
0.5
1
1.5
2
2.5
3

Figure 9 - Correlation between Mismatch Repair prechemotherapy immunoscores

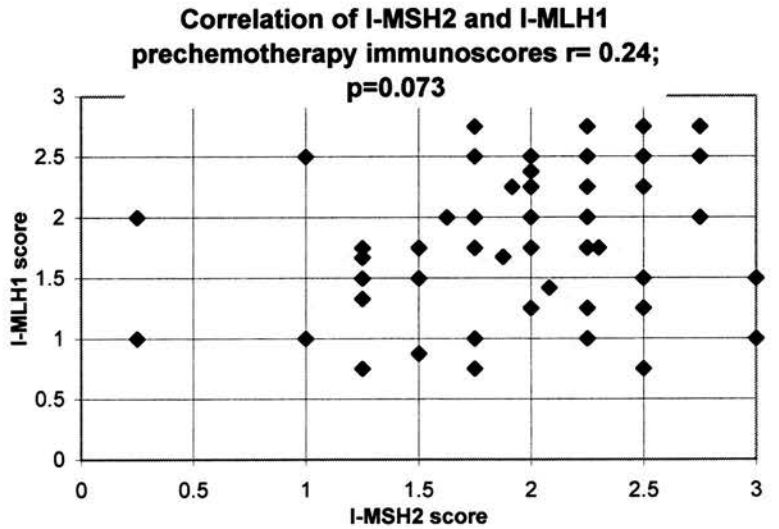
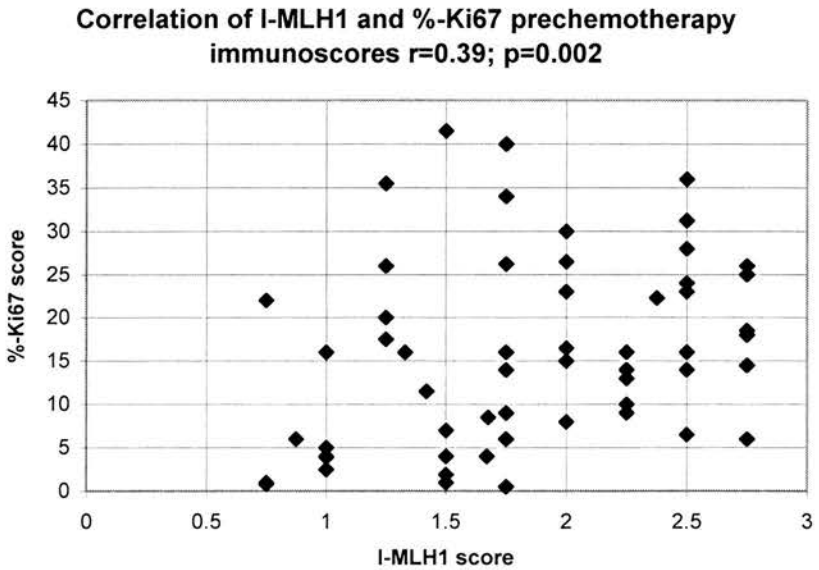
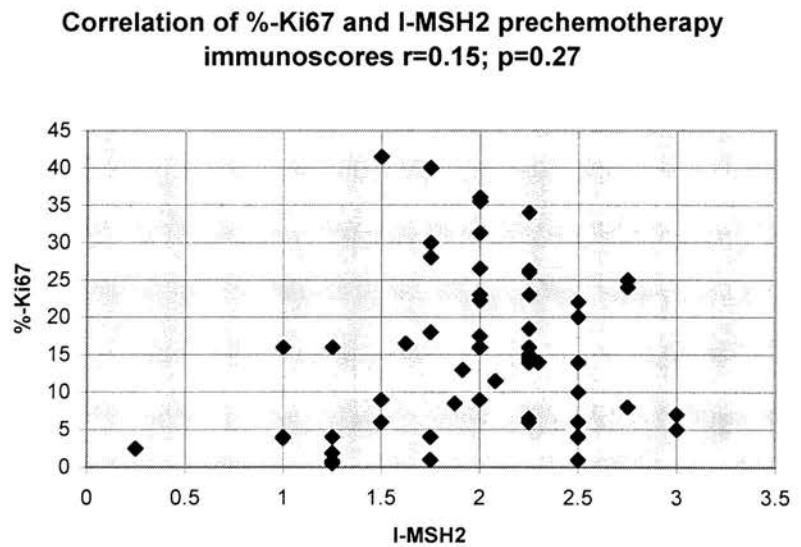
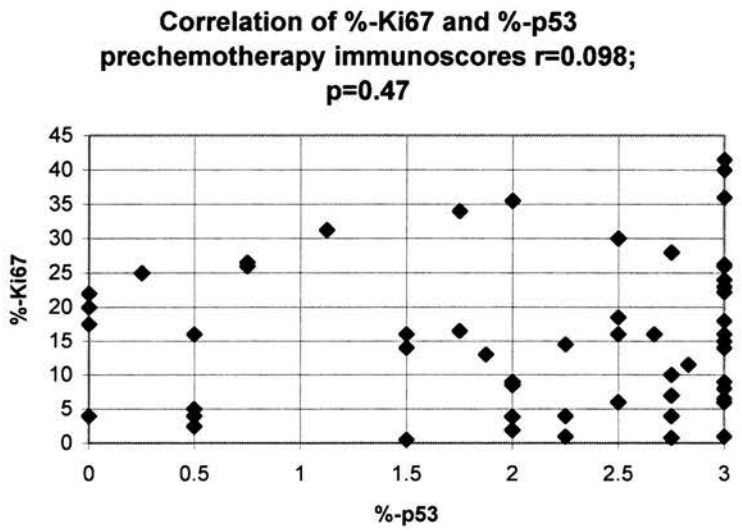
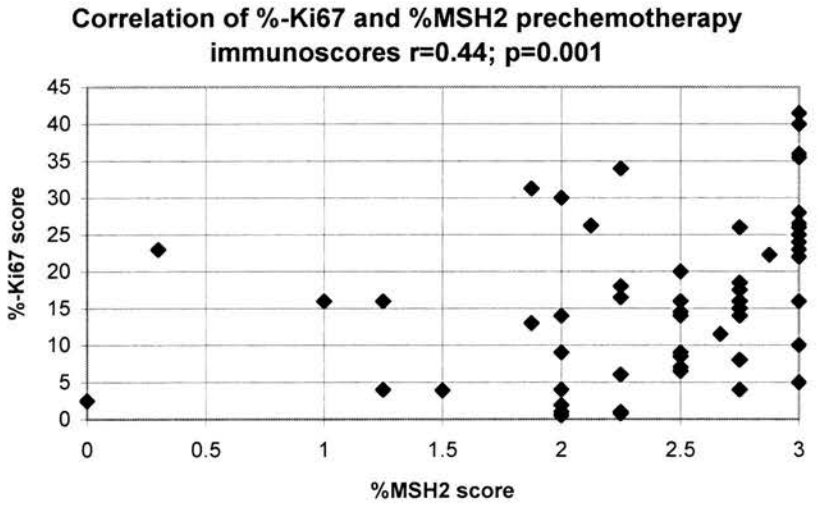


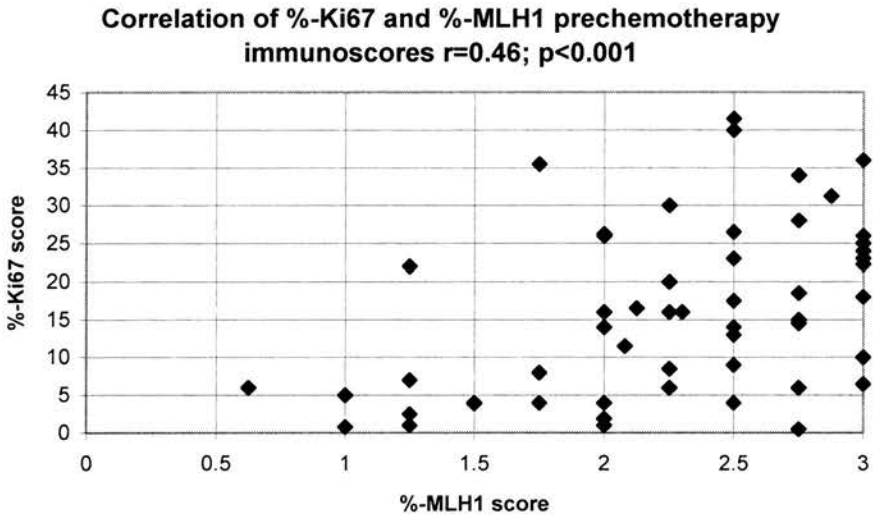
Figure 10 - Correlation of MMR protein expression and Ki67






Correlation of %-Ki67 and %-p53
prechemotherapy immunoscores r=0.098;
p=0.47
%-Ki67
45
40
35
30
25
20
15
10
5
0
0
0.5
1
1.5
2
2.5
3
%-p53


Correlation of %-Ki67 and %MSH2 prechemotherapy immunoscores r=0.44; p=0.001
%-Ki67 score
%MSH2 score
0
5
10
15
20
25
30
35
40
45
0
0.5
1
1.5
2
2.5
3


Correlation of %-Ki67 and %-MLH1 prechemotherapy immunoscores r=0.46; p<0.001
%-Ki67 score
%-MLH1 score
0
5
10
15
20
25
30
35
40
45
0
0.5
1
1.5
2
2.5
3

D - CONCLUSIONS OF IMMUNOSCORES

- Adequate positive and negative controls for IHC were obtained only 3/4 of the time.

- Only IHC runs with concurrent adequate controls were used for scoring.

- The distribution of MSH2, and MLH1 scores are skewed with more scores at the top of the range than the lower (0-3 range).

- The distribution of p53 was bimodal with two peaks at 0 and 3 scoring.

- Only 7% of blocks were negative for MSH2 and/or MLH1 (i.e. mismatch repair deficient).

- The distribution of Ki67 scores are skewed to the lower range with a tail of higher scores.

- There is some heterogeneity of IHC staining between and within tissues but this was not found to be significant on a limited study and was less than 0.5 of a score for MLH1, MSH2 and p53.

- Intensity and percentage scores appear related to one another for MLH1, p53 and MSH2. This suggests there is some coregulation of expression of these proteins both within a single cell and between surrounding cells.

- I-MLH1, %-MLH1 and %-MSH2 scores showed a significant association with Ki67 score. This suggests expression of these MMR proteins is related to proliferation.

- There is a weak association of %-MLH1 and %-MSH2 suggesting coregulation of expression.

- There appears to be no correlation between mismatch repair protein expression and p53 expression.

Chapter 7 – CORRELATION OF IMMUNOSCORES WITH CLINICAL FEATURES AND OUTCOME

A – CORRELATION OF IMMUNOSCORES WITH STAGE, RESIDUAL DISEASE AND PERFORMANCE STATUS

Because of the known prognostic significance of stage, residual disease, histology and performance status (Voest, van Houwelingen et al. 1989) we examined the relationship of these variables with the proteins studied. These were examined by Kruskal-Wallis one-way analysis of variance. The results are shown in table 35 and figure 11. There was a statistically significant association with stage of the tumour and %-scores for MLH1, MSH2 and Ki67 (p=0.0092, 0.0049 and 0.0054 respectively), and MSH2 I-score (p=0.019). The MLH1 I-score approached significance with stage (p=0.062). This suggests there is increased proliferation and also increased expression of MMR proteins with advanced stage (3 or 4). Scores for p53 did not show any association with stage. All immunoscores showed no association with performance status or residual disease.

Table 35 – Association of immunoscores with clinical prognostic factors in ovarian cancer

Each cell shows the median value and the interquartile range in brackets. Significant values ($p<0.05$) are shown in bold.

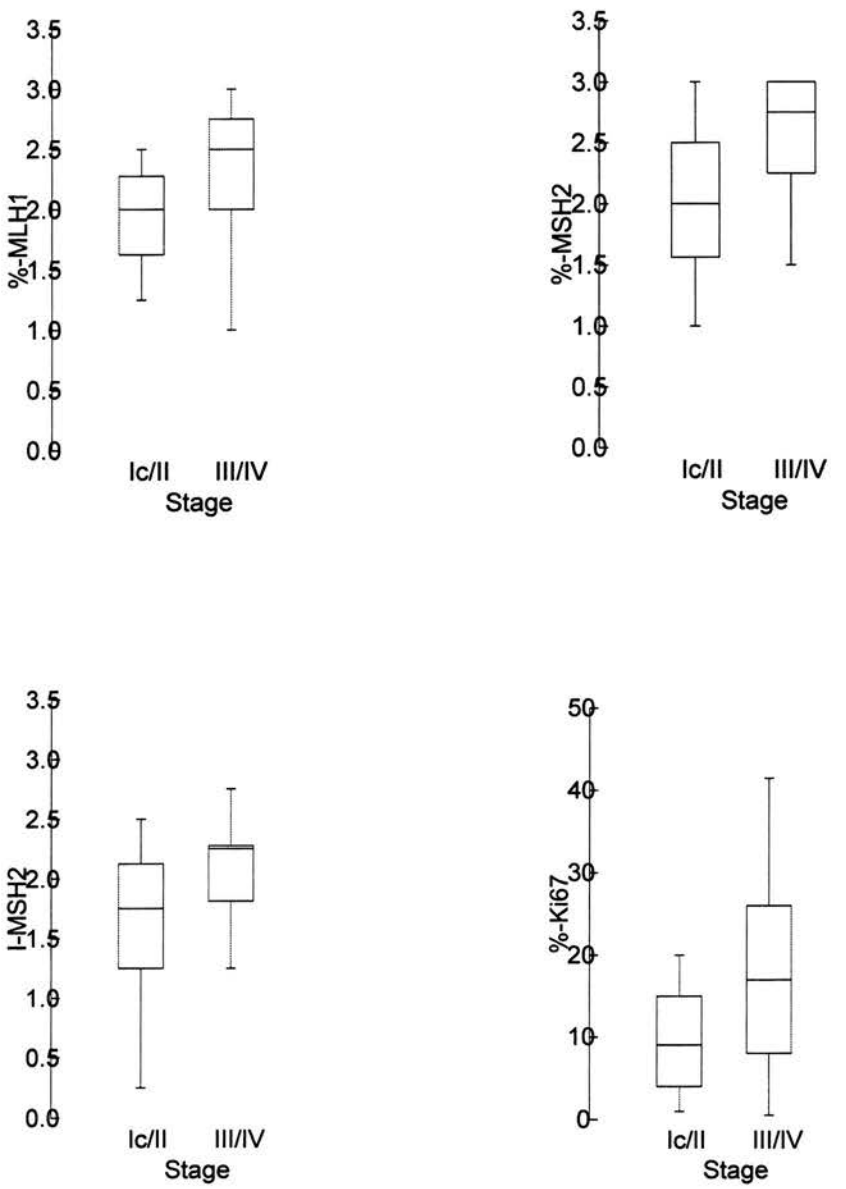
STAGE			
	Ic/II (n=15)	III/IV (n=43)	p-value
I-MLH1	1.50 (1.00-2.25)	2.00 (1.50-2.50)	0.062
%-MLH1	2.00 (1.63-2.28)	2.50 (2.00-2.75)	**0.0092**
I-MSH2	1.75 (1.25-2.13)	2.25 (1.81-2.28)	**0.019**
%-MSH2	2.00 (1.56-2.50)	2.75 (2.25-3.00)	**0.0049**
I-p53	2.00 (1.38-2.81)	2.67 (1.00-3.00)	0.51
%-p53	2.25 (1.00-3.00)	2.75 (1.75-3.00)	0.35
%-Ki67	9 (4-15)	17 (8.13-26)	**0.0054**

PERFORMANCE STATUS			
	0 (n=19)	1 or 2 (n=39)	p-value
I-MLH1	1.75 (1.29-2.25)	1.75 (1.46-2.44)	0.86
%-MLH1	2.25 (2.00-2.63)	2.25 (2.00-2.75)	0.86
I-MSH2	1.92 (1.75-2.25)	2.25 (1.69-2.28)	0.19
%-MSH2	2.25 (2.00-2.75)	2.50 (2.19-3.00)	0.23
I-p53	2.00 (1.50-3.00)	2.67 (1.00-3.00)	0.98
%-p53	2.50 (1.69-3.00)	2.67 (1.63-3.00)	0.95
%-Ki67	13 (4-17)	16 (8-26)	0.16

RESIDUAL DISEASE			
	<2cm (n=20)	>2cm (n=38)	p-value
I-MLH1	2.00 (1.25-2.31)	1.75 (1.50-2.34)	0.97
%-MLH1	2.25 (2.00-2.56)	2.38 (2.00-2.75)	0.77
I-MSH2	2.13 (1.75-2.31)	2.00 (1.66-2.25)	0.89
%-MSH2	2.38 (1.72-2.81)	2.50 (2.16-2.84)	0.4
I-p53	2.50 (1.63-3.00)	2.50 (1.00-3.00)	0.54
%-p53	2.63 (1.25-3.00)	2.38 (1.75-3.00)	0.72
%-Ki67	16 (5-21)	15 (7-25)	0.65

Figure 11– Early stage is associated with lower %-MLH1, MSH2, Ki67 and I-MLH1 immunoscore

Shown by Box plot: Line inside box = median, Box = inter-quartile range, Error bars = 95% centiles for the entire population, stars = outliers



B – CORRELATION OF IMMUNOSCORES WITH HISTOLOGY

Because of the previous finding of a correlation of MSI and endometrioid histology (Fujita, Enomoto et al. 1995; King, Carcangiu et al. 1995), we investigated any possible correlation of immunoscores with histology using Kruskal-Wallis one-way analysis of variance. There was a significant correlation with MMR protein expression and histology (see table 36 and figure 12). Only 3 histologies (not including endometrioid) were included in the analysis due to the small number of patients with other histological types. For MLH1 there was a higher median %- and I-score for the papillary type compared with serous cystadenocarcinoma or adenocarcinoma ($p=0.0076$ and 0.038 respectively; $n=49$). For MSH2 a higher median I-score was seen with both serous cystadenocarcinoma and papillary adenocarcinoma compared to adenocarcinoma ($p=0.023$; $n=49$). This suggests a consistently lower expression of MMR proteins in the adenocarcinoma histological subtype.

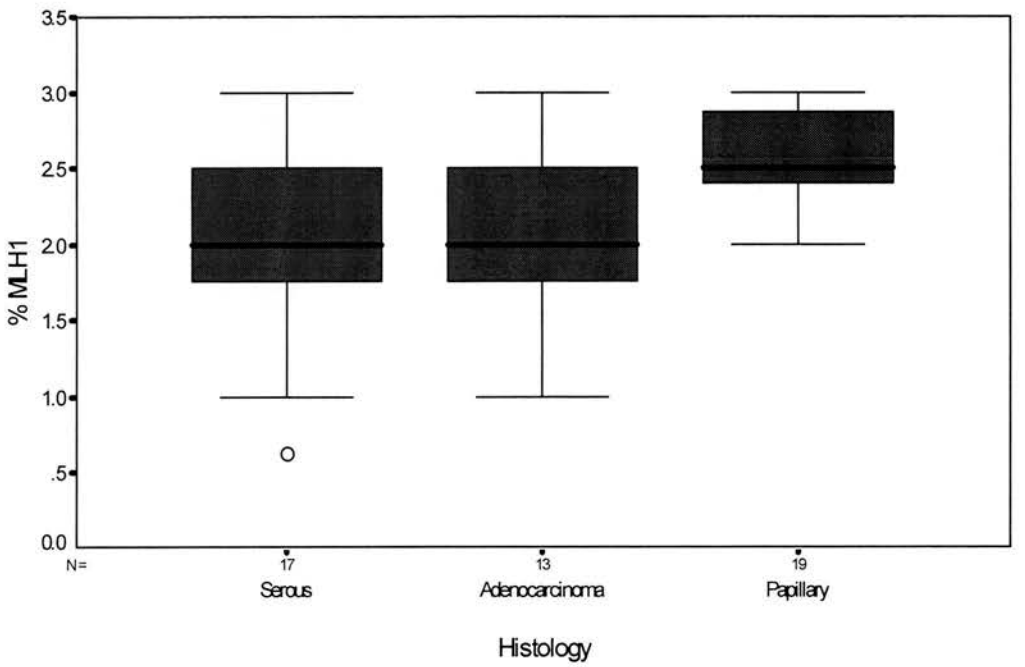
Table 36 – Association of histology with immunoscores

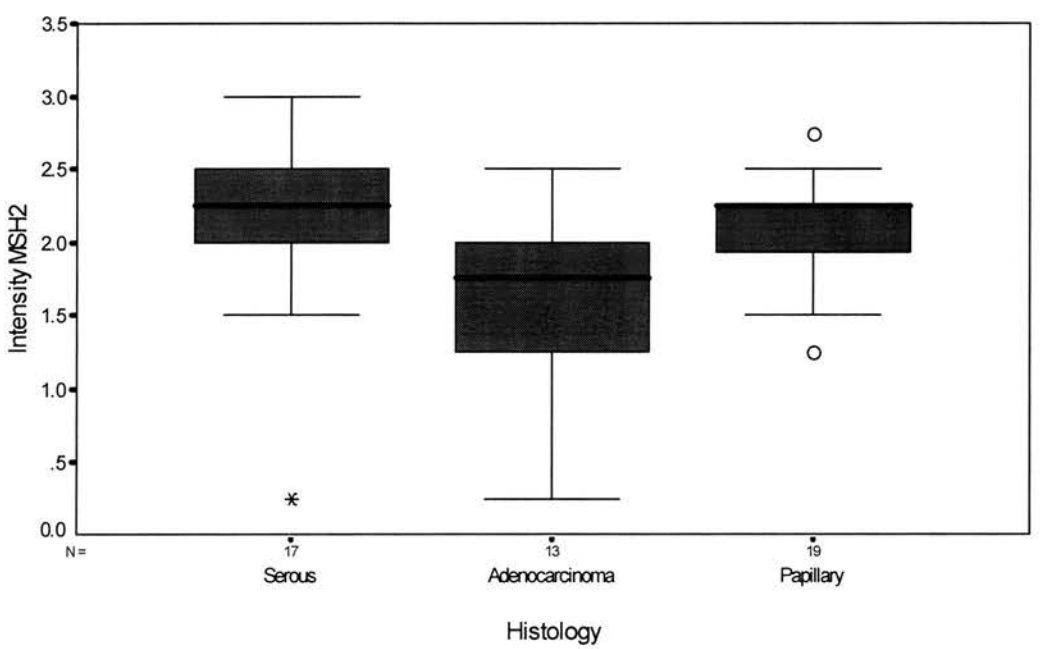
Each cell entry is the'median (interquartile range)'. Significant p values (<0.05) are shown in bold.

	Serous Cysta-denocarcinoma	Adenocarcinoma	Papillary adenocarcinoma	**p-value**
N=	18	16	20	
I-MLH1	1.75 (1.25-2.25)	1.75 (1.00-2.00)	2.25 (1.63-2.50)	**0.038**
%-MLH1	2.00 (1.75-2.50)	2.00 (1.75-2.00)	2.50 (2.30-3.00)	**0.0076**
I-MSH2	2.25 (2.00-2.50)	1.75 (1.25-2.00)	2.25 (1.88-2.25)	**0.023**
%-MSH2	2.67 (2.25-3.00)	2.25 (2.00-2.88)	2.50 (2.25-3.00)	0.58
I-p53	2.88 (2.00-3.00)	2.00 (1.00-3.00)	2.75 (1.00-3.00)	0.85
%-p53	2.83 (2.00-3.00)	2.00 (2.00-3.00)	2.75 (1.75-3.00)	0.60
%-Ki67	10 (6-16)	16 (6-27)	19 (9-28)	0.088

Figure 12 - Correlation of immunoscores with histology

Shown by Box plot: Line inside box = median, Box = inter-quartile range, Error bars = 95% centiles, circles = outliers





C - CORRELATION OF IMMUNOSCORES WITH SURVIVAL

All patients had a histological diagnosis of ovarian adenocarcinoma and were treated with primary cisplatin combination chemotherapy. The median overall survival of the patients is 29 months. A Cox proportional hazards model, stratified for type of chemotherapy, was used to determine whether immunohistochemistry results for MMR, p53 or Ki67 were correlated with survival. Results are shown in table 37.

On univariate analysis both early stage (IC or II) and residual disease <2cm were good prognostic features (Hazard Ratio (HR)=2.66; 95%CI=1.14-6.19; p=0.014 and HR=2.47; 95%CI=1.15-5.31; p=0.014 respectively). The Kaplan-Meier survival curves for stage and residual disease are shown in figures 13 + 14. On univariate analysis only the MSH2 %-score was significant (HR=1.66; 95%CI=1.02-2.72; p=0.042) with an increased percentage of cells expressing MSH2 correlating with poor survival. However this association was absent on multivariate analysis and review of the data suggested that the significance of the MSH2 %-score on univariate analysis was due to the association between high % MSH2 expression and late stage, which is itself a poor prognostic feature.

Multivariate analysis for survival, stratified for chemotherapy regimen, and using a model including established prognostic factors of stage, extent of residual disease and performance status was performed for each of the immunohistochemistry scores. Of the immunoscores only the MLH1 I-score was statistically significant (HR=0.46; 95%CI=0.22-0.94; p=0.031). This implies that after correcting for clinical prognostic

features, a low intensity of staining for MLH1 pre-chemotherapy is an independent predictor of poor overall survival. Kaplan-Meier survival curves showing the patient population separated by both MLH1 I-score and stage are shown in figure 15.

Table 37 - Results of univariate and multivariate analysis for survival (n=58)

	Univariate		Multivariate	
	Hazard Ratio*	95% Confidence Interval	Hazard Ratio*	95% Confidence Interval
Stage	**3.02**	**1.30 - 7.00**		
Residual Disease	**2.29**	**1.13 - 4.64**		
Performance Status	1.41	0.70 - 2.81		
I-MLH1	0.76	0.42 - 1.39	**0.46**	**0.22 – 0.94**
%-MLH1	1.00	0.56 - 1.78	0.71	0.38 – 1.33
I-MSH2	0.93	0.54 - 1.60	0.69	0.39 – 1.22
%-MSH2	**1.66**	**1.02-2.72**	1.28	0.72 – 2.27
I-p53	0.88	0.63 - 1.23	0.78	0.56 – 1.11
%-p53	0.94	0.68 - 1.31	0.78	0.54 – 1.12
%-Ki67	1.03	0.99 - 1.07	1.03	0.98 – 1.07

*Multiplicative change in hazard for a unit increase in the corresponding variable

Significant values shown in bold

Figure 13 - Kaplan Meier Curve of cause specific survival stratified for residual disease (n=58)

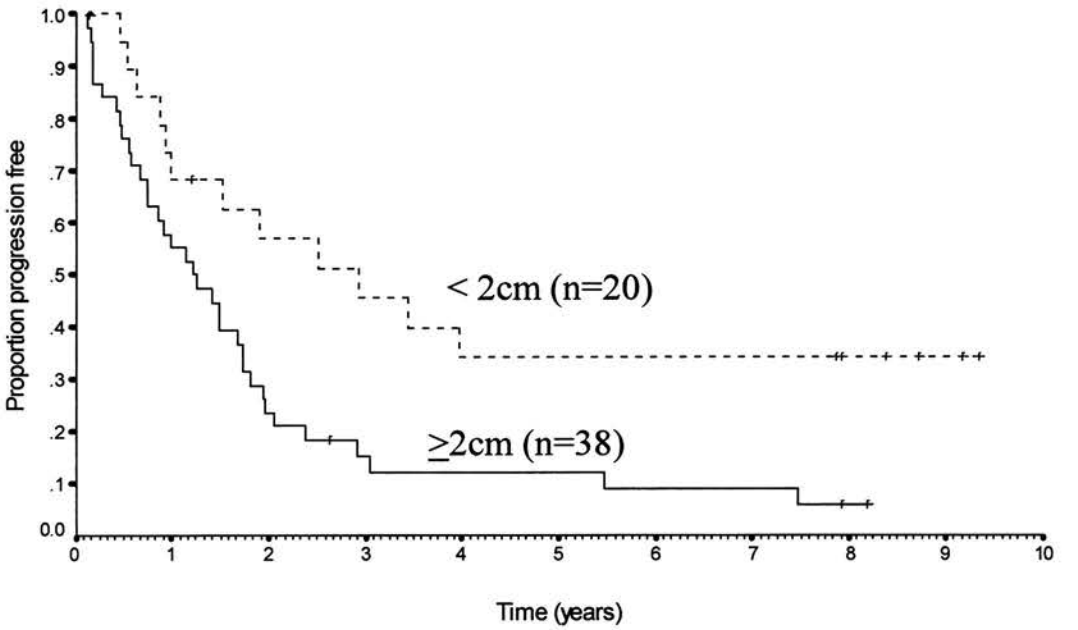


Figure 14 - Kaplan Meier Curve of cause specific survival stratified for stage (n=58)

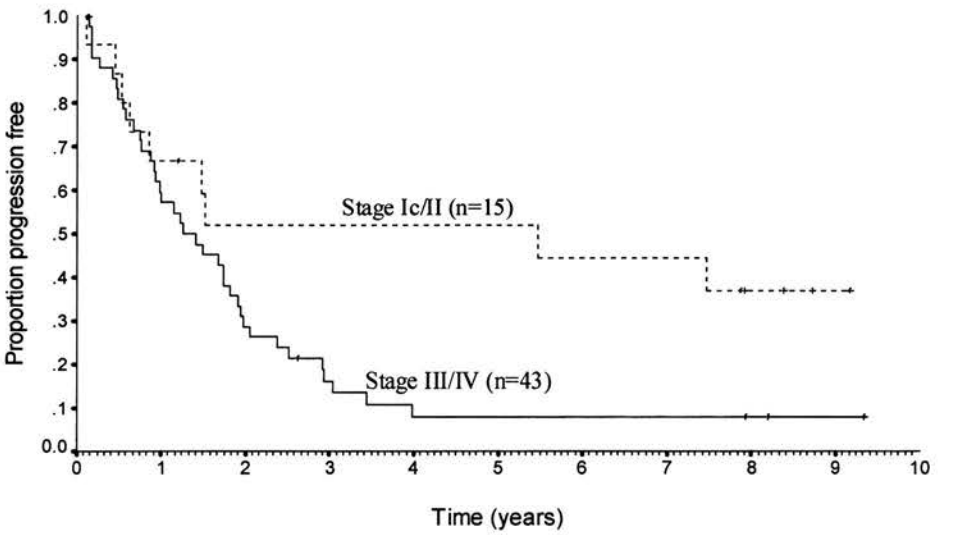
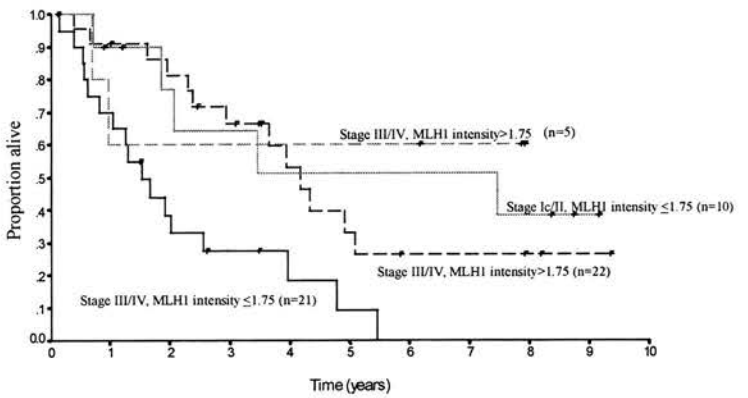


Figure 15 – Kaplan-Meier Curve of cause specific survival stratified for stage and intensity MLH1 score



Loss of Mismatch Repair Expression and survival

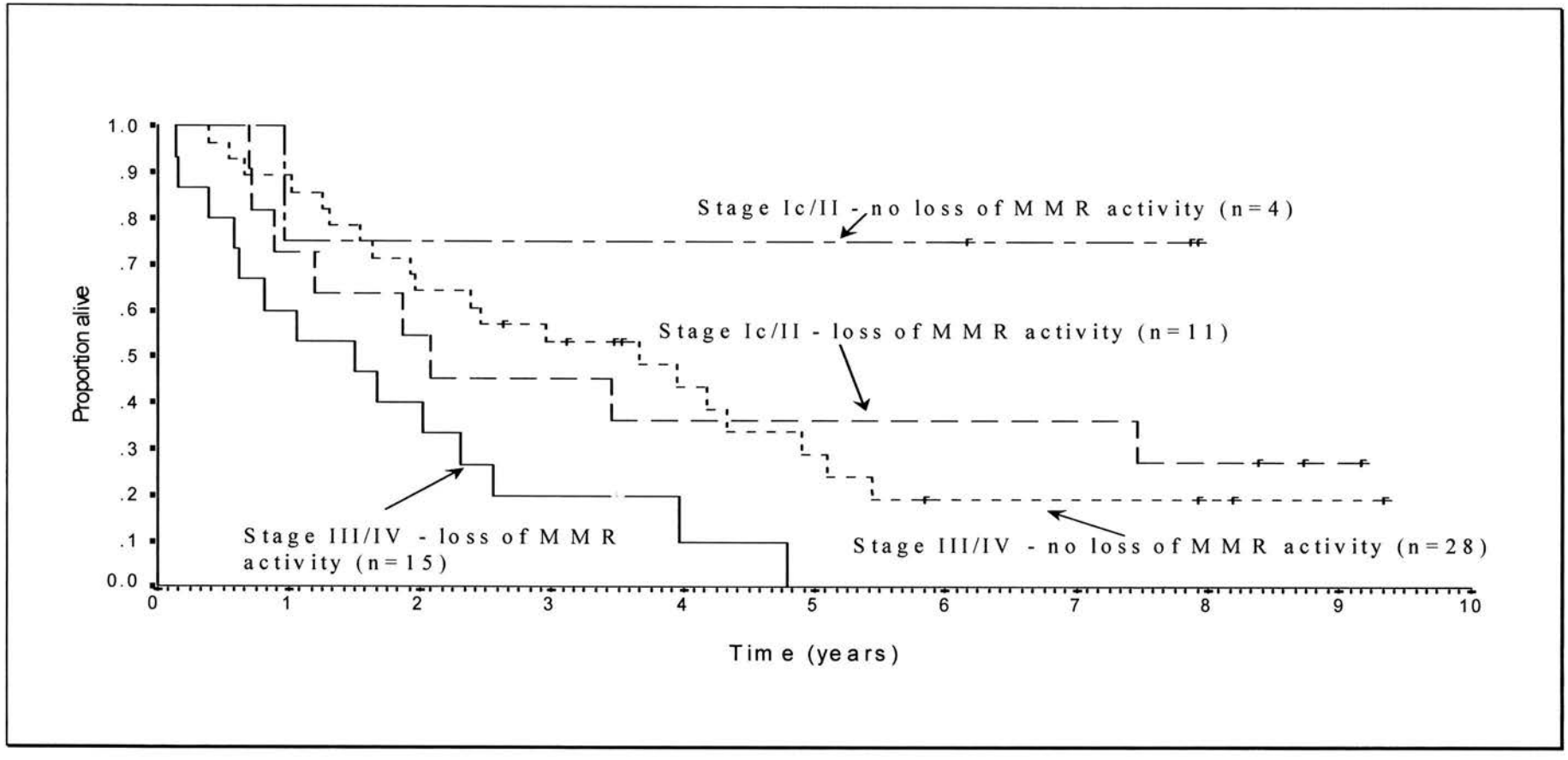
As a reduction in either MLH1 or MSH2 could lead to loss of function of DNA mismatch repair we compared 2 groups of patients for survival. Those with intact mismatch repair (defined as intensity score for both MLH1 and MSH2 of over 1.5) and those with 'loss of I-MMR' (i.e. I-MLH1 <=1.5 and/or I-MSH2 <= 1.5). We used intensity scores as these were the most significant in the above analysis. Interestingly adding in those patients with a low intensity of MSH2 as well as low I-MLH1 identified more accurately a subgroup of poor prognosis patients than I-MLH1 alone, shown in table 38. If a patient has either a reduction in I-MLH1 and/or I-MSH2 they are 3.64 times more likely to die based on a Cox multivariate analysis (including stage, residual disease and performance status; stratified for chemotherapy) compared to patients with intact MMR. The median survival for patients with 'loss of I-MMR' is 21 months (647.5 days) compared to 39 months (1201 days) for intact I-MMR, shown in figure 16.

Table 38 – Univariate and multivariate analysis for survival and loss of MMR status

Survival	Univariate analysis		Multivariate analysis	
	Hazard Ratio	95% confidence intervals	Hazard Ratio	95% confidence intervals
Loss of I-MMR*	1.67	0.87 – 3.21 p=0.12	**3.64**	**1.66 – 7.99** **p=0.0012**

*Loss of I-MMR is defined as I-MLH1<= 1.5 and/or I-MSH2 <=1.5

Figure 16 - Kaplan-Meier curve for survival by I-MMR status and stage (p=0.0012)



D – CORRELATION OF IMMUNOSCORES WITH TIME TO PROGRESSION

The median time to progression (TTP) for these patients was 18 months. A Cox proportional hazards model, stratified for type of chemotherapy, was used to examine whether immunohistochemistry results for MMR, p53 or Ki67 were correlated with time to progression. Results are shown in table 39.

On univariate analysis both early stage (i.e. stage 1C or II) and residual disease <2cm were good prognostic features (HR=2.25; 95%CI=1.05-4.84; p=0.027 and HR=2.63; 95%CI=1.31-5.31; p=0.004 respectively). On univariate analysis only the MSH2 %-score was significant (HR=1.67; 95%CI=1.02-2.73; p=0.024). This means patients with an increased percentage of cells expressing MSH2 showed a shorter time to progression.

On multivariate analysis using a model including established prognostic factors of stage, extent of residual disease and performance status, none of the prechemotherapy immunoscores predicted for TTP, again suggesting that the significance of the MSH2 %-score on univariate analysis was due to its association with stage.

Again, as a reduction in either MLH1 or MSH2 could lead to loss of function of DNA mismatch repair, we compared 2 groups of patients for time to progression. Those with intact mismatch repair (defined as intensity score for both MLH1 and MSH2 of over 1.5) and those with 'loss of I-MMR' (i.e. I-MLH1 <=1.5 and/or I-MSH2 <= 1.5).

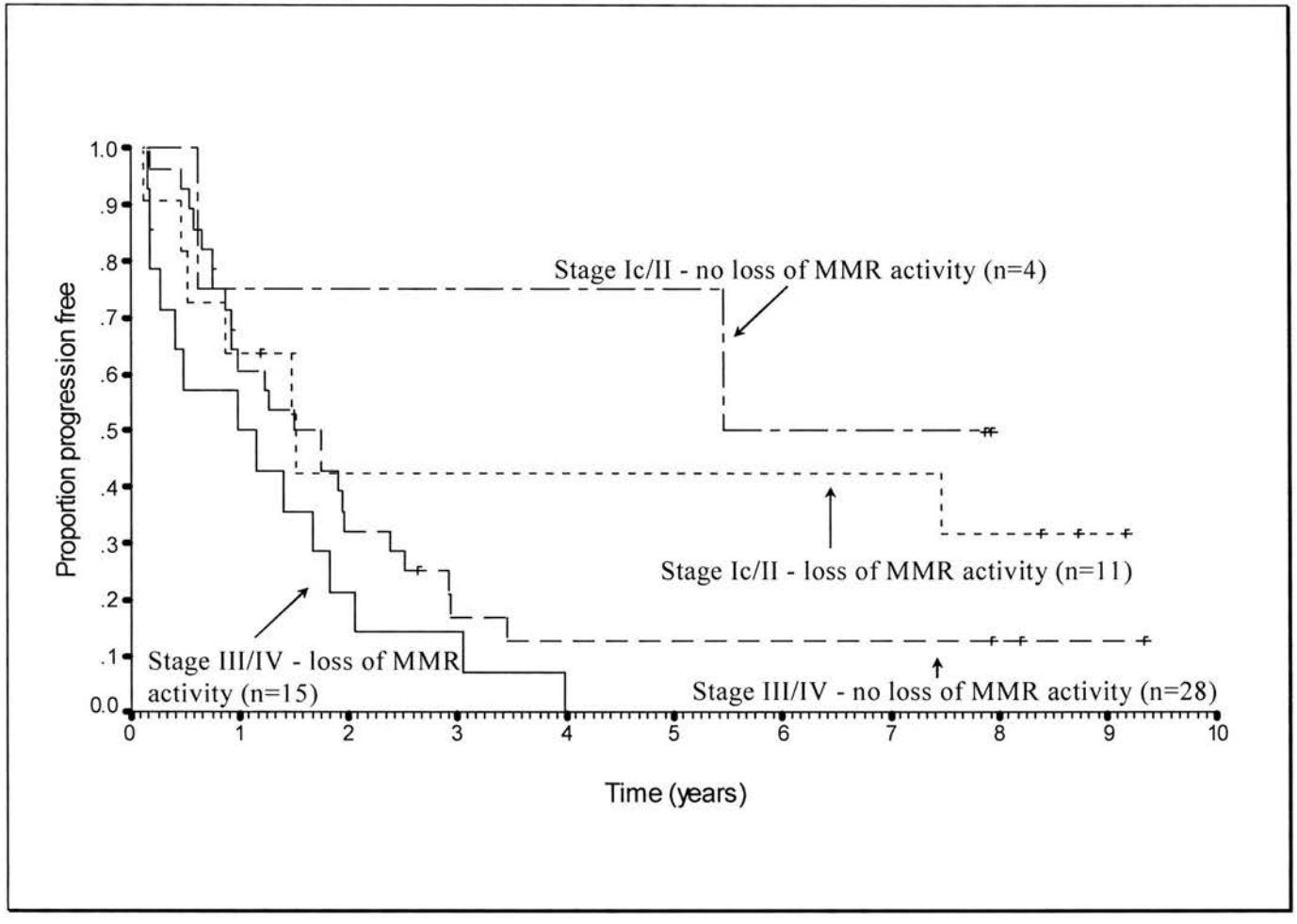
We used intensity scores as these were the most significant for survival. Interestingly adding in those patients with a low intensity of MSH2 as well as low I-MLH1 identified a subgroup of poor prognosis patients. If a patient has either a reduction in I-MLH1 and/or I-MSH2 they are 2.37 times more likely to have disease progression based on a Cox multivariate analysis (including stage, residual disease and performance status; stratified for chemotherapy) compared to patients with intact MMR. Shown in figure 17.

Table 39 - Results of Cox analysis with Time to Progression (n=58)

Significant values shown in bold	UNIVARIATE		MULTIVARIATE	
	Hazard Ratio*	95% CI	Hazard Ratio*	95% CI
Stage	**2.25**	**1.04 – 4.84**		
Residual Disease	**2.63**	**1.31 - 5.31**		
Performance Status	1.33	0.69 - 2.54		
I-MLH1	0.89	0.52 - 1.52	0.65	0.35 - 1.19
%-MLH1	1.08	0.63 - 1.86	0.86	0.48 - 1.54
I-MSH2	1.03	0.62 - 1.70	0.85	0.50 - 1.44
%-MSH2	**1.67**	**1.02 - 2.73**	1.36	0.80 - 2.32
I-p53	0.97	0.71 - 1.32	0.90	0.66 - 1.23
%-p53	1.03	0.77 - 1.40	0.92	0.66 - 1.27
%-Ki67	1.03	0.99 - 1.06	1.02	0.98 - 1.05
I-MLH1<=1.5 and/or I-MSH2<=1.5	1.39	0.76-2.55	**2.37**	**1.18-4.76** **p=0.016**

*Multiplicative change in hazard for a unit increase in the corresponding variable

Figure 17 - Kaplan-Meier curve for progression free survival stratified for loss of I-MMR and stage



E – CORRELATION OF IMMUNOSCORES WITH RESPONSE (tumour shrinkage) TO CHEMOTHERAPY

Of the 58 patients, 22 had no evaluable disease or did not have repeat assessments. 7 patients had stable disease or progressive disease (19%) while the remaining 29 had a response (CR or PR=81%). To investigate whether clinical factors or MMR, p53 or Ki67 immunohistochemistry predicted for response we used a logistic regression model. Response (CR or PR) was compared to no response (SD or PD) and was again stratified for the type of chemotherapy given. Immunoscores and clinical factors were examined individually and then the immunoscores were examined in a model containing 3 prognostic factors. The results are shown in table 40.

There was no significant correlation of response with stage, performance status, or residual disease. Of all of the immunoscores only the p53 %-score suggested any association with response (multivariate analysis HR=2.89; 95%CI=0.86-9.73; p=0.059). This suggests that tumours with an increased percentage of cells expressing p53 showed a trend towards a better response to treatment, shown in figure 18. On this occasion loss of I-MMR (i.e. I-MLH1 <=1.5 and/or I-MSH2 <= 1.5) showed no association with response on univariate or multivariate analysis.

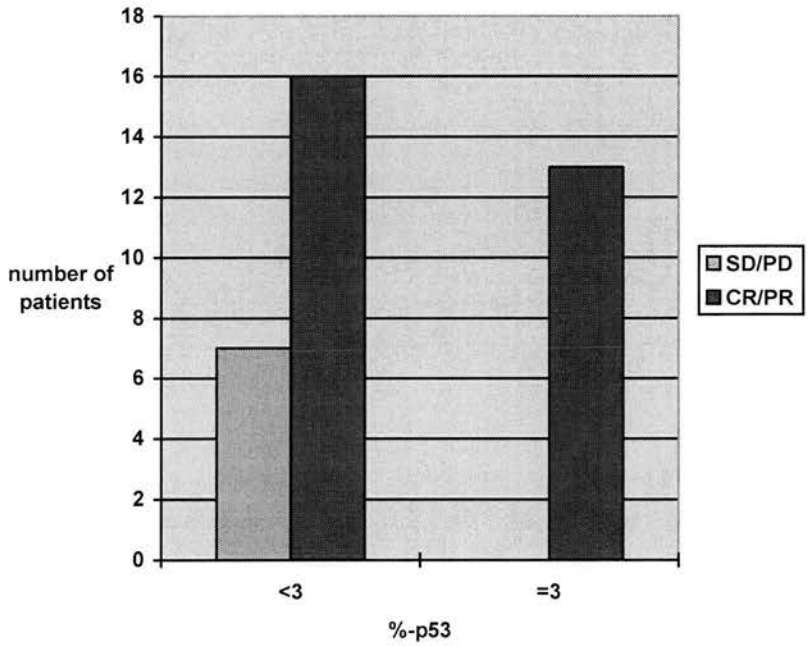
Another surrogate measurement of response to chemotherapy in ovarian cancer is the rise or fall of the tumour marker CA 125 during treatment. Unfortunately there were insufficient measurements in these patients to permit this to be analysed.

Table 40 - Results of logistic regression for association with response

	Univariate	Multivariate	
	Odds Ratio*	Odds Ratio*	95% Confidence Interval
Stage	0.57		
Residual Disease	0.83		
Performance Status	0.34		
I-MLH1	1.73	1.93	0.36 - 10.40
%-MLH1	0.89	0.99	0.19 - 5.10
I-MSH2	0.98	0.92	0.20 - 4.16
%-MSH2	0.58	0.61	0.11 - 3.43
I-p53	1.46	1.62	0.61 - 4.35
%-p53	2.40	2.89	0.86 – 9.73
%-Ki67	1.04	1.05	0.94 - 1.16
I-MLH1<=1.5 and/or I-MSH2<=1.5	0.28	0.22	0.03-1.81

*Multiplicative change in odds in favour of response for a unit increase in the corresponding variable. Significant values shown in bold.

Figure 18 - Significance of %-p53 score and response



F - CONCLUSIONS OF CORRELATION OF IMMUNOSCORES WITH CLINICAL FEATURES AND OUTCOME

- Advanced stage (stage III/IV) is correlated with increasing expression of MLH1, MSH2 and Ki67. As stage itself is a known prognostic factor any prognostic study of MMR proteins will need to correct for stage.

- There was no association of MLH1, MSH2, Ki67 or p53 with residual disease or performance status.

- There was an association of lower expression of MMR proteins in adenocarcinoma histological subtype.

- Loss of mismatch repair, as defined by an intensity score below the mid-range value of 1.5 for either MLH1 or MSH2, was shown to predict for poor overall survival and decreased time to progression on multivariate analysis. This is clinical evidence supportive of the *in-vivo* and *in-vitro* data discussed in the introduction suggesting that loss of MMR is important in resistance to platinum based chemotherapy.

- On multivariate analysis for survival reduced intensity of MLH1 staining was found to be statistically significantly associated with poorer overall survival. This suggests that prechemotherapy MLH1 protein expression is associated with clinical outcome in this group of ovarian cancer patients treated with platinum based chemotherapy.

- As expected advanced stage and residual disease >2cm were associated with poor overall survival and reduced time to progression.

- On univariate analysis increasing %-MSH2 score was significantly associated with poor overall survival and reduced time to progression but this was due to its association with advanced stage and lost significance on multivariate analysis. This supports the need to have clinical prognostic factors accounted for in any study such as this.

- There was no statistically significant association of any immunostain with response to chemotherapy but the numbers included were small in keeping with the difficulty of obtaining accurate response data in ovarian cancer patients.

G – MULTIPLE IMMUNOSCORES FOR THE SAME PATIENT

A – PAIRED PRE AND POST CHEMOTHERAPY SAMPLES

Paired blocks of tissue were obtained from 26 patients taken at the initial diagnostic laporotomy prior to chemotherapy and at a second operation following chemotherapy. The patient characteristics are shown in table 41. There was an excess of patients with more advanced stage compared to the patient characteristics for prechemotherapy samples (n=58) but no other significant differences. Patients with advanced disease are more likely to have residual disease and, therefore, a second operation. The timing of the second operation varied. 18 of the 26 patients had repeat operations within 12 months of their initial laporotomy (***postchemotherapy residual*** *paired samples*). 12 of the 26 patients had repeat operations at least 12 months after their initial laporotomy (***relapsed*** *paired samples*). Four of the 26 patients had multiple operations both within and after a 12 month period following diagnosis. The numbers of paired samples are small and therefore any conclusions are limited due to sample size.

Table 41 – Patient characteristics for paired samples

(N=26)		N=	%
Chemotherapy	Cisplatin (50)/Cyclophosphamide	10	38
	Cisplatin (100)/Cyclophosphamide	8	31
	Cisplatin (100)/Cyclophosphamide	3	11
	Cisplatin (75)/ Taxol	2	8
	Cisplatin (75)/ Cyclophosphamide	3	11
Stage	IC	1	4
	II	3	11
	III	19	73
	IV	3	11
Performance Status	0	8	31
	1	15	58
	2	3	11
Residual Disease	<2cm (1)	8	31
	2cm or greater (2)	18	69

A (I) - Difference in paired sample scores (n=26)

There was no significant difference found between pre and post chemotherapy immunoscores, (using Wilcoxon matched pairs signed ranks test), as shown in table 42.

Table 42 – Difference in pre and post chemotherapy immunoscores

N=26	Mean score **pre chemo**	Mean score **post chemo**	No. of patients with **increased** score post chemo	No. of patients with **decreased** score post chemo	No. of patients with **the same** score post chemo	p-value
I-MSH2	2.08	1.99	11	11	4	0.51
%-MSH2	2.44	2.38	10	13	3	0.39
I-p53	2.20	2.07	5	10	11	0.47
%-p53	2.33	2.25	8	8	10	0.90
I-MLH1	1.96	1.81	10	15	1	0.35
%-MLH1	2.35	2.27	8	10	8	0.78
%-Ki67	17.91	16.62	8	15	1	0.41

A (II) - Correlation of change in immunoscore postchemotherapy with survival

To determine whether altered expression of either MMR proteins, p53 or Ki67 following cisplatin containing chemotherapy had an impact on survival, a Cox univariate and multivariate analysis was performed using the difference in score (pre-post chemotherapy immunscores). The multivariate analysis included the known clinical prognostic variables of initial performance status, residual disease and stage. Data was censored for one case that progressed early and the analyses were stratified for chemotherapy regimen (i.e. cisplatin dose). The median overall survival for these patients was 34 months. The results are shown in table 43. No significant difference was found suggesting that the change in MMR, p53 or Ki67 protein expression after chemotherapy, as measured by immunohistochemistry, does not predict for overall survival in these patients.

Table 43 - Results of univariate and multivariate analyses for survival with difference in immunoscore postchemotherapy (n=26)

	UNIVARIATE ANALYSIS		MULTIVARIATE ANALYSIS	
	Hazard Ratio	95% CI	Hazard Ratio	95% CI
I-MSH2	0.70	0.40-1.24	0.65	0.35-1.19
%-MSH2	1.13	0.70-1.84	1.25	0.73-2.13
%-Ki67	1.00	0.96-1.05	1.00	0.95-1.05
I-p53	1.06	0.60-1.85	1.10	0.61-1.98
%-p53	0.84	0.52-1.34	0.79	0.48-1.32
I-MLH1	0.82	0.39-1.74	0.79	0.27-2.32
%-MLH1	1.02	0.58-1.78	0.81	0.33-1.99
Residual Disease	0.97	0.31-3.06		
Stage	1.02	0.27-3.92		
Performance Status	1.32	0.43-4.04		

A (III) - Correlation of change in immunoscore postchemotherapy with time to progression (TTP)

Similarly, to investigate whether altered expression of either MMR proteins, p53 or Ki67 following cisplatin containing chemotherapy had an impact on time to progression a Cox univariate and multivariate analysis was performed using the difference in score (pre-post chemotherapy) and the known clinical prognostic variables of initial performance status, residual disease and stage. Data was censored for one case that progressed early and the analyses were stratified for chemotherapy regimen. The median time to progression for these patients was 19 months. The results are shown in table 44.

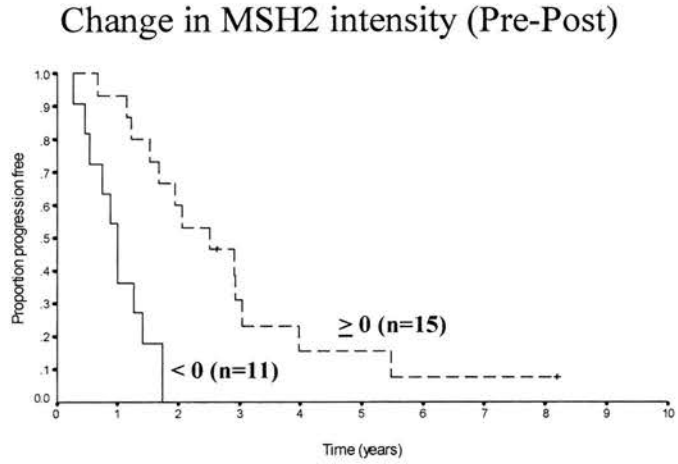
On both univariate and multivariate analysis there was a significant association found with the change in intensity score for MSH2 (I-MSH2) and TTP. The likelihood ratio p values were 0.035 for the univariate and 0.011 for the multivariate analyses. 11 patients showed an increase in I-MSH2 score after chemotherapy with a median TTP of 361 days. 15 patients showed a reduced or stable I-MSH2 score following chemotherapy with a longer median TTP of 834 days. This suggests that a loss of MSH2 expression after chemotherapy with a cisplatin containing regimen is associated with a prolonged time to progression. This is shown on the Kaplan Meier curve in figure 19.

Table 44 - Results of univariate and multivariate analyses for time to progression with difference in immunoscore postchemotherapy (n=26)

TIME TO PROGRESSION	UNIVARIATE ANALYSIS		MULTIVARIATE ANALYSIS	
	Hazard Ratio	95% CI	Hazard Ratio	95% CI
I-MSH2	**0.59**	**0.35-0.997**	**0.52**	**0.30-0.90**
%-MSH2	1.2	0.79-1.83	1.37	0.88-2.12
%-Ki67	0.998	0.96-1.03	0.99	0.95-1.03
I-p53	0.99	0.60-1.62	1.04	0.63-1.72
%-p53	0.92	0.60-1.41	0.88	0.56-1.36
I-MLH1	0.68	0.35-1.34	0.60	0.23-1.56
%-MLH1	1.10	0.65-1.85	0.90	0.42-1.94
Residual Disease	0.93	0.35-2.46		
Stage	1.14	0.31-4.24		
Performance Status	1.55	0.53-4.49		

Significant values shown in bold.

Figure 19 - Kaplan-Meier curve for time to progression stratified for increase or decrease in I-MSH2 score postchemotherapy (n=26)



This shows that patients with a loss of intensity of MSH2 staining post chemotherapy have a longer time before their ovarian cancer shows progressive disease.

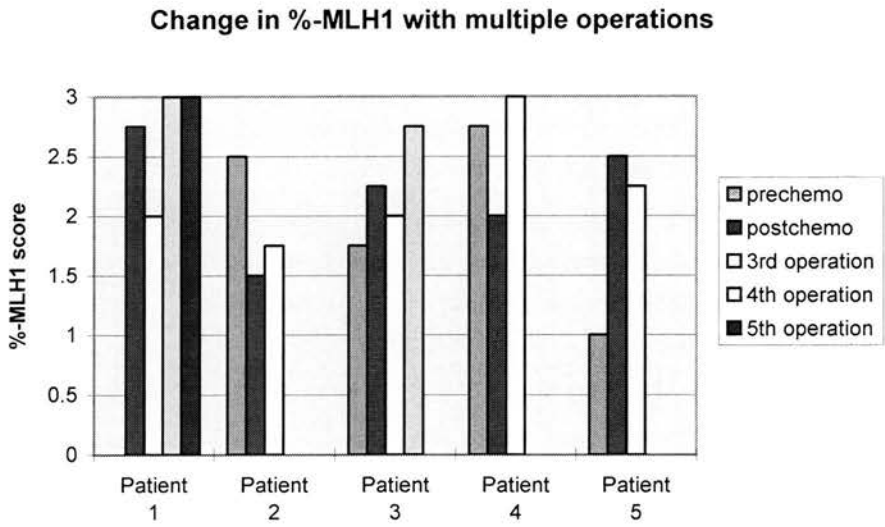
A (III) - Conclusions from paired samples

There is no consistent difference in immunoscores pre and post chemotherapy. However, patients with a loss of I-MSH2 staining post chemotherapy show a significantly longer TTP (p=0.011 on multivariate analysis). However the number of patients examined with paired samples is small, and includes both residual and relapsed pairs. Any firm conclusions are therefore very limited.

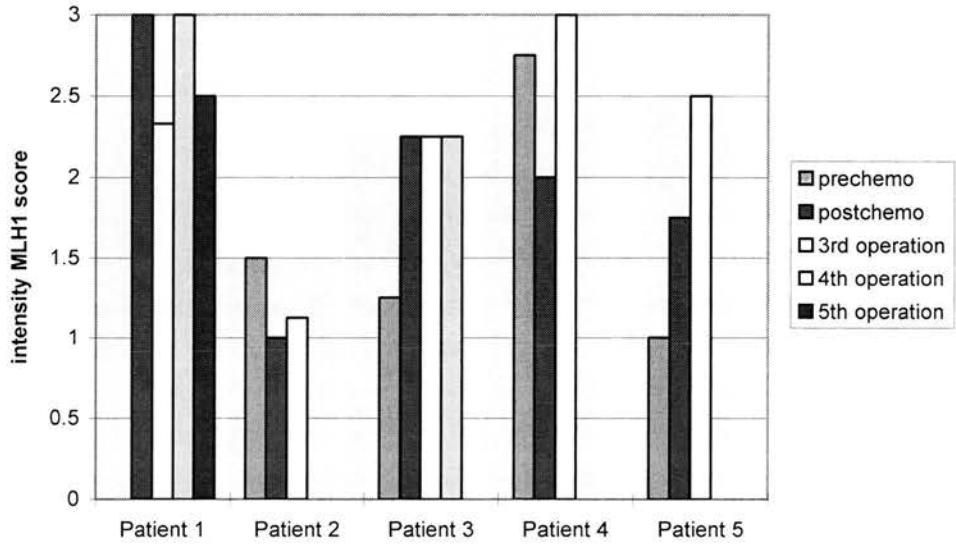
B – IMMUNOSCORES FROM MULTIPLE OPERATIONS

Multiple blocks were obtained from 5 patients who had more than 2 operations for ovarian cancer. In one of these no pre-chemotherapy block was obtained. No clear pattern for change over time with either MMR, p53 or ki67 expression was observed in this very small number of patients. Results are shown in bar charts in figure 20 below.

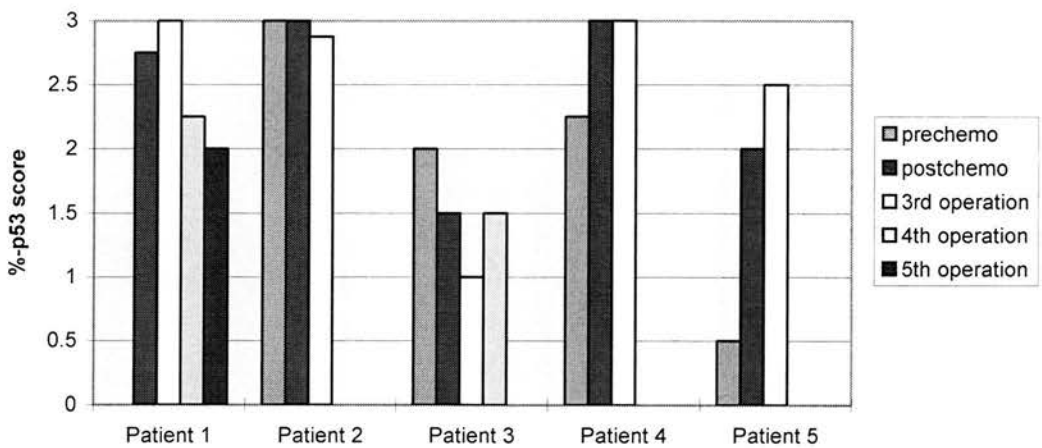
Figure 20 – Bar charts of change in immunoscores with multiple operations



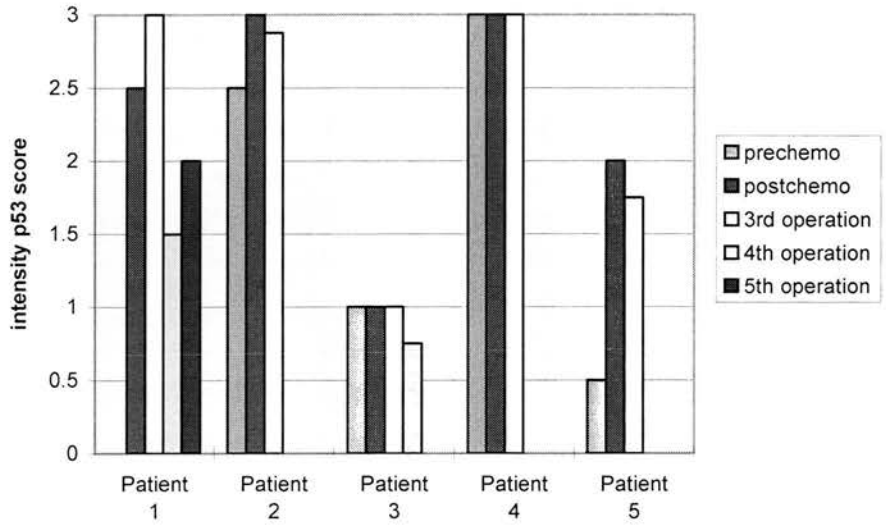
Change in Intensity MLH1 with multiple operations (n=5)



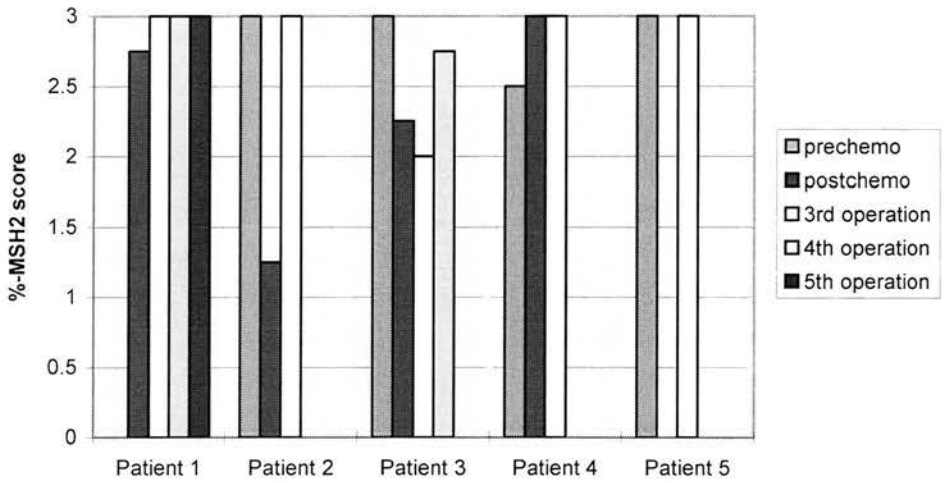
Change in %-p53 with multiple operations (n=5)



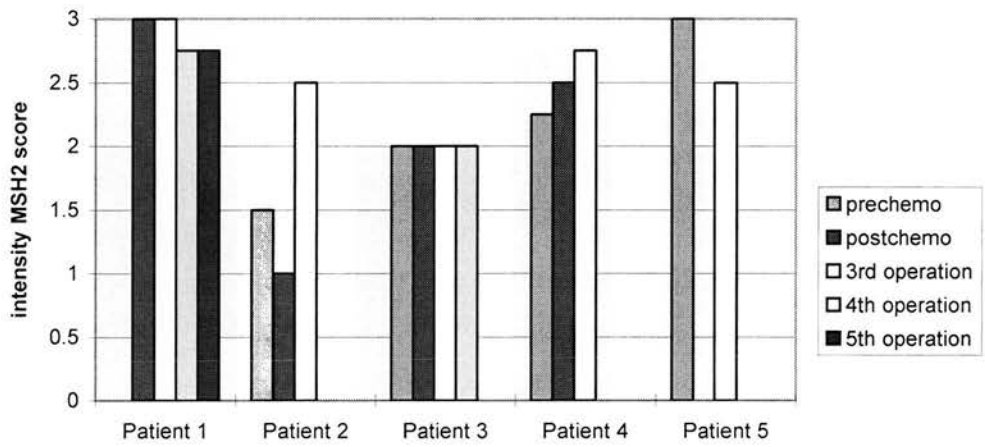
Change in l-p53 with multiple operations (n=5)



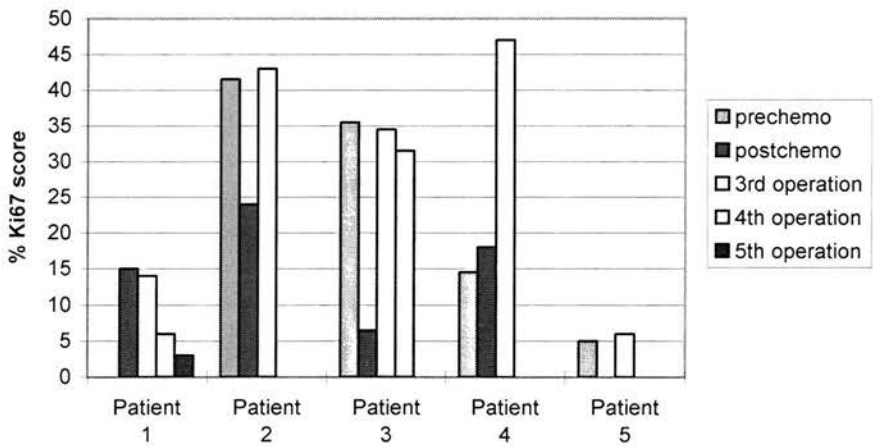
Change in %-MSH2 with multiple operations (n=5)



Change in I-MSH2 with multiple operations (n=5)



Change in Ki67 score with multiple operations (n=5)



H – ADDITIVE HISTOSCORE

In view of the wealth of literature on combined histoscores (see introduction) we investigated whether an additive Histoscore (H) calculated by; Intensity score (0 to 3) + Percentage score (0 to 3) for MLH1, MSH2 and p53 would provide any additional prognostic information (i.e. from 0 to maximum 6). On univariate and multivariate Cox analysis for survival and progression free survival there were no new significant results and indeed the significant results for I-MLH1 on multivariate analysis for survival were lost, shown in table 45.

Table 45 – Results of analysis of additive histoscores with outcome

Survival	Univariate		Multivariate	
	Hazard Ratio*	95% Confidence Interval	Hazard Ratio*	95% Confidence Interval
H-MLH1	0.93	0.68 – 1.26	0.74	0.52 – 1.05
H-MSH2	1.09	0.82 – 1.43	0.93	0.69 – 1.25
H-p53	0.96	0.80 – 1.14	0.88	0.74 – 1.06
Progression Free Survival				
H-MLH1	0.99	0.75 – 1.31	0.85	0.62 – 1.16
H-MSH2	1.12	0.86 – 1.45	1.00	0.76 – 1.33
H-p53	1.00	0.86 – 1.17	0.95	0.81 – 1.12

*Multiplicative change in hazard for a unit increase in the corresponding variable

Likewise for correlation with histology the additive histoscore loses any significance for MSH2 and are less significant for H-MLH1 compared to %-MLH1 alone (p=0.015 and 0.0076 respectively), shown in table 46.

Table 46 – Results of additive histoscore and histology

Histology	N=	Mean Rank		
		H-MLH1	H-MSH2	H-p53
Serous cystadenocarcinoma	18	20.74	28.29	27.06
Adenocarcinoma	16	19.73	18.46	23
Papillary adenocarcinoma	20	32.42	26.53	24.53
Chi-Square (2df)		8.45	3.89	0.66
p=		**0.015**	0.14	0.72

By logistic regression none of the H-scores showed a significant association with response to chemotherapy which is identical to the results for I-scores and %-scores alone, shown in table 47.

Table 47 – Results of additive histoscore with response

Correlation with response	H-MLH1	H-MSH2	H-p53
p value	0.77	0.72	0.20

The only histoscore that has shown an increased sensitivity compared with %-score or I-score alone is that for the correlation of MSH2 with stage. Both I-MSH2 and %-MSH2 show a correlation with stage (p=0.019 and 0.0049 respectively) and the additive histoscore is more significant than either alone (p=0.0034). This does not hold true for MLH1, shown in table 48.

Table 48 – Results of additive histoscore with clinical factors

2-tailed p values for Mann Whitney U-test	Stage	Performance Status	Residual Disease
H-MLH1	**0.029**	0.94	0.99
I-MLH1	**0.062**	0.86	0.97
%-MLH1	**0.0092**	0.86	0.77
H-MSH2	**0.0034**	0.12	0.8
I-MSH2	**0.019**	0.19	0.89
%-MSH2	**0.0049**	0.23	0.4
H-p53	0.36	0.95	0.51

Examination of the Spearman rank correlation coefficients between histoscores shows no added sensitivity for H-scores compared to I-scores or %-scores, shown in table 36. In particular the significant correlation of H-MLH1 with H-MSH2 (r=0.27; p=0.042) is no greater than the correlation of %-MLH1 and %-MSH2 (r=0.30;

p=0.022). Similarly, the correlation of %-Ki67 with H-MLH1 and H-MSH-2 (r=0.44; p=0.001 and r=0.34; p=0.009 respectively) is no better than that with %-MLH1 and %-MSH2 (r=0.46; p<0.001 and r=0.44; p=0.001 respectively).

Conclusions for use of additive histoscore

Apart from the single correlation of stage and H-MSH2 additive score there is no evidence to support the use of an additive histoscore instead of separate Intensity and Percentage score in these three antibodies.

Table 49 – Results of correlation of additive histoscores

	H-MSH2	H-p53	H-MLH1
Ki67	**0.34;** **0.009**	0.066; 0.63	**0.44;** **0.001**
H-MSH2	NA	0.016; 0.90	**0.27;** **0.042**
I-MSH2	NA	0.062; 0.64	0.22; 0.10
%-MSH2	NA	-0.051; 0.70	0.23; 0.077
H-p53	0.016; 0.90	NA	0.15; 0.25
I-p53	0.0005; 0.997	NA	0.12; 0.35
%-p53	0.019; 0.89	NA	0.17; 0.21
H-MLH1	**0.27;** **0.042**	0.15; 0.25	NA
I-MLH1	0.25; 0.062	0.21; 0.12	NA
%-MLH1	**0.29;** **0.03**	0.11; 0.42	NA

- Spearmank Rank correlation coefficients (r) shown above
- p values shown below in italics
- Significant ($p \leq 0.05$) values shown in bold
- NA = not applicable (scores are derived from each other)

Chapter 8 -

MMR IMMUNOHISTOCHEMISTRY IN ENDOMETRIAL SPECIMENS

A - INTRODUCTION

Endometrial carcinoma is the most common non-colorectal carcinoma in women affected by HNPCC, and microsatellite instability has been observed both in the inherited form and in approximately 20% of presumed sporadic endometrial carcinomas (Risinger, Berchuck et al. 1993; Burks, Kessis et al. 1994). The aim of this small study was to examine the expression of hMLH1 and hMSH2, compared to p53 and Ki67 in the normal stages of endometrial development, atypical endometrium and endometrial carcinoma.

B - MATERIALS AND METHODS

The immunohistochemistry for the four antibodies was performed as previously described in methods chapter. Scoring was done by only one observer (DM) due to the difficulty in interpretation of pathology of endometrial tissues. On this occasion scoring for Ki67 was performed as per MMR and p53 on a scale of 0 to 3 for both intensity and percentage. Each antibody was run independently on two separate slides for each case and each slide scored blindly on separate occasions. The final scores are a mean of the two results. Five different patients' slides were used to examine the

range of expression of MMR proteins from normal to carcinoma. These slides were of normal proliferative endometrium, normal early secretory, normal late secretory, hyperplastic endometrium and endometrial carcinoma.

C - RESULTS

As only 10 slides were examined in total no attempt has been made to examine statistical significance in this small sample.

Immunoscores are shown in table 50 and figure 21 below. Proliferation was high in all phases of endometrial tissue examined apart from the late secretory phase. This is the normal phase of specialisation of the endometrial tissue. By contrast MMR proteins hMLH1 and hMSH2 both showed a fall in expression in both the early and late secretory phase of differentiation. In the abnormal hyperplastic endometrium hMLH1 was increasingly expressed, unlike hMSH2 and p53. With the frankly carcinomatous endometrium both MMR proteins were strongly expressed. P53 was also overexpressed suggesting that the mutant form were present.

Figure 21 – Additive histoscores of immunohistochemistry in endometrial samples

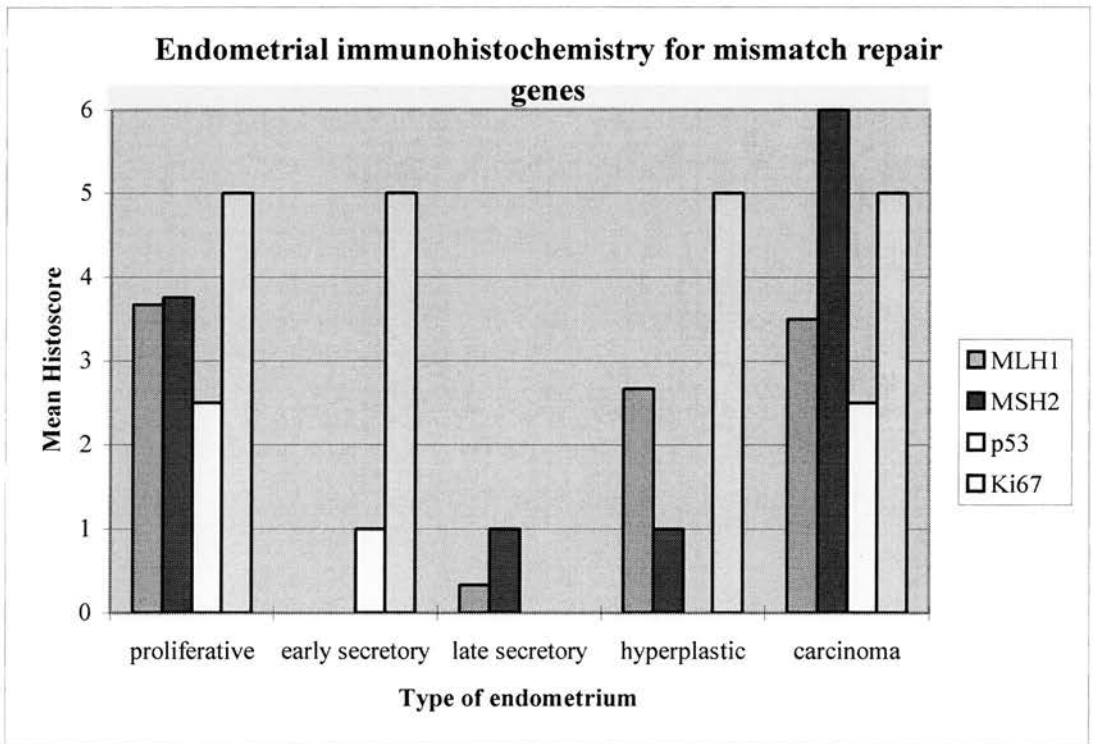


Table 50 – Results of immunohistochemistry scores of endometrial samples (n=5)

Type of endometrium	Proliferative	Early secretory	Late secretory	Hyperplastic	Carcinoma
I-MLH1	1.67	0	0.33	1	1.5
%-MLH1	2	0	0	1.67	2
H-MLH1	3.67	0	0.33	2.67	3.5
I-MSH2	2	0	0.75	0.5	3
%-MSH2	1.75	0	0.25	0.5	3
H-MSH2	3.75	0	1	1	6
I-p53	1	0.5	0	0	1
%-p53	1.5	0.5	0	0	1.5
H-p53	2.5	1	0	0	2.5
I-Ki67	3	3	0	3	3
%-Ki67	2	2	0	2	2
H-Ki67	5	5	0	5	5

D - DISCUSSION

This small study suggests that up regulation of MMR proteins occurs in malignant compared to normal differentiated secretory endometrium. This is in keeping with a much larger study comparing hMSH2 expression in malignant (n=40) and secretory normal endometrium (n=15) (Freidrich, Villena-Heinsen et al. 1999). They found hMSH2 was consistently and strongly up regulated in endometrial carcinoma compared to normal endometrium in the secretory phase ($p<0.001$). Interestingly they showed no association of hMSH2 staining with Ki67 staining.

It has been shown that MSI in sporadic endometrial carcinomas is not due to mutations in hMSH2, hMLH1, hPMS1 or hPMS2 (Katabuchi, van Rees et al. 1995; Lim, Tester et al. 1996). The MSI may be due to hypermethylation of the promoter silencing the MMR gene. In 14 MSI endometrial carcinomas no gene defects in MLH1 or MSH2 were detected but hypermethylation of the MLH1 promoter regions was identified in 10 case (71%) (Gurin, Federici et al. 1999). However, immunohistochemistry for hMLH1 and hMSH2 is intact in MSI positive endometrial tumours (Lim, Tester et al. 1996) suggesting that there is some expression of MLH1. It may be hMSH3 or hMSH6 that is important in these tumours to account for the MSI phenotype. It has been shown that patients with more advanced endometrial cancer (Stage III or IV or with lymph node metastases) are more likely to have MSI. Also no MSI was found in 26 patients with precancerous atypical endometrial hyperplasia (Ohwada, Suzuki et al. 1999). This suggests that MSI is a late finding in endometrial carcinoma.

Chapter 9 - DISCUSSION

1. PRINCIPAL FINDINGS

a) Key clinical finding

We have shown that reduced expression of the DNA mismatch repair genes MLH1 and/or MSH2, by intensity of immunohistochemistry, in pre-chemotherapy samples from patients with ovarian cancer who are subsequently treated with cisplatin containing chemotherapy, independently predicts for poor progression free and overall survival.

b) Other Clinical Findings in ovarian cancer patients

- Reduced expression of MLH1 prechemotherapy independently predicts for poor overall survival.
- Ki67 and p53 expression does not predict outcome in this group of ovarian cancer patients.
- Advanced stage of tumour is associated with an increased percentage of cells expressing Ki67, MSH2 and MLH1 and increased intensity of MSH2. There is no correlation with immunoscores and residual disease or performance status.
- Reduced expression of MMR proteins is seen in the adenocarcinoma histological subtype.
- There was no significant difference found in pre and post chemotherapy expression of MLH1, MSH2, p53 or Ki67 in our group of patients.

- An increase in expression of MSH2 post chemotherapy independently predicts for poor progression free survival but not overall survival.

c) Technical Findings

- It is possible to immunostain paraffin embedded tissues for MMR proteins, MLH1 and MSH2 in a consistent manner.

- Using a four-point scale for both intensity and percentage of cells stained it is possible to obtain good correlation in inter-observer, intra-observer and intra-slide scores.

- The intensity of staining is closely correlated to the percentage of cells stained.

- Immunostaining for MLH1 and MSH2 are correlated with each other and increase with increasing proliferation as measured by Ki67.

- MMR immunostaining is not correlated with p53 immunostaining.

- There was no advantage of an additive histoscore (I+%) and the correlation of MMR and survival was lost.

2. STENGTHS AND WEAKNESSES OF THIS STUDY

In an editorial on statistical issues in cancer research Simon and Altman describe the key features of a study on prognostic factors for valid conclusions to be drawn (Simon and Altman 1994). These are the first set of guidelines for such studies and provide a framework to assess the results of different prognostic factor studies that are often

inconsistent or contradictory. Using these 11 guidelines the current study has been examined for possible strengths and weaknesses.

1. *Intra- and inter-laboratory reproducibility of assays should be documented.*

Intra-laboratory reproducibility of the immunostaining was provided by staining 2 slides for each case, on separate occasions (the intra-slide variability). The kappa scores ranged from 0.583 for intensity of MLH1 to 0.905 for intensity of p53. For immunohistochemistry it is also important to state the inter- and intra-observer variability for the scoring system used. We found this to have a kappa score consistently above 0.6 for all antibodies. The inter-laboratory reproducibility of the staining has not been tested. The use of both positive and negative control slides in each run developed for this study e.g. A2780 +ve and CP70 –ve for MLH1 should help in quality assurance between laboratories.

2. *Laboratory assays should be performed blinded to clinical outcome.*

The 2 scorers for the immunohistochemistry were both blinded to clinical outcome and to each other's results.

3. *A clear inception cohort of patients should be assembled with few (e.g.<15%) patients non-evaluable due to missing material or data. The referral pattern and eligibility criteria should be described so that generalisability of results can be evaluated.*

The study was potentially subject to selection bias as patients were chosen on the availability of histological material, although all patient samples that could be retrieved were included. Patients entered into the 3 trials included came from the entire West of Scotland region and 12 different pathology laboratories were involved

in retrieving blocks for the current study. Comparing the patient characterisits in our study (n=58) to those in the study by Kaye et al. (n=159) (Kaye, Paul et al. 1996) we found no difference in the stage, presence of residual disease or performance status between the two cohorts (by X^2 analysis; $p>0.05$; data not shown) suggesting that our group of patients was representative.

4. *Treatment (or absence of treatment) should be standardised or randomised and accounted for in the analysis and interpretation.*

Patients received 4 different chemotherapy regimens within 3 separate trials. We stratified the Cox analyses for chemotherapy to remove the effect of regimen, including dose of cisplatin, from the analysis. All patients were managed by the same multidisciplinary team after initial diagnosis. Any future studies should be on a larger group of patients all treated with the same protocol for surgery (both initially and after chemotherapy) and chemotherapy regimen. However, prospective validation studies in ovarian cancer may be complicated by the inclusion of taxanes in treatment protocols, since taxanes are believed to induce cytotoxicity in a manner independent of MMR status (Fink, Aebi et al. 1998).

5. *Hypotheses to be tested should be stated in advance. The hypotheses should include specification of end points, cut-off values for prognostic variables, subsets of patients, treatment, standard prognostic factors or classifications to be used that are relevant to the hypothesis.*

The hypothesis that loss of MMR proteins, in particular MLH1, may lead to cisplatin resistance and therefore poor outcome in ovarian cancer patients was stated in the aims of this thesis which were submitted to University of Edinburgh in advance as part of the MD application. The end points of overall survival, progression free

survival and response were also stated. The immunoscores individually were examined as continuous variables and therefore cut-off points were not used. However, the cut-off point for loss of intensity of MMR staining (i.e. I-MLH1<=1.5 and/or I-MSH2<=1.5) was not stated in advance and can therefore be criticised.

6. *The number of patients and number of 'events' should be sufficiently large that statistically reliable results are obtainable. Statistical power calculations that incorporate the number of hypotheses to be tested and the appropriated subset of patients (e.g. node-negative) for each hypotheses should be described.*

In keeping with many prognostic studies we are guilty of not stating the statistical power and number of patients required in advance of the study. There were no patient subset analyses performed on this group of patients. The sample size calculations become particularly important when considering 'negative' results that may be merely 'uninformative' due to small sample size. The positive results from our study are thus less of a concern. The observation in the present study that loss of MMR predicts for progression-free and overall survival, but not for response is perhaps surprising, although defining response in ovarian cancer is fraught with difficulties. The number of patients that could be assessed for response was small, with only 7 patients having stable or progressive disease and thus limiting the power of the statistical analysis.

7. *Analyses should test whether new assays add predictiveness once outcome is adjusted for the effect of standard prognostic factors.*

We were very careful in this study to include known prognostic variables for ovarian cancer in a multivariate analysis. The only variables we did not adjust for were histology and FIGO grade of tumour both of which have not been shown to be a consistent prognostic variable.

8. *The analyses should be adjusted for the number of hypotheses to be tested.*

Although the present study was designed to specifically examine the prognostic value of MLH1 and MSH2, there is a danger that examination of multiple parameters can lead to a statistical correlation by chance. Clearly the current study needs independent validation. Indeed a significant value should only be accepted if the computed p-valued is less that 0.05 x C, where C = the number of comparisons. In our study there were 8 variables tested (I-MLH1, %-MLH1, I-p53, %-p53, I-MSH2, %-MSH2, %-Ki67 and Loss of I-MMR) against outcome. The finding that loss of MMR is associated with poor overall survival survives this scrutiny with a p value of (0.0012 x 8) = 0.0096. However the significance of loss of MMR with progression free survival and I-MLH1 with overall survival is lost.

9. *Analyses should be based on prespecified cut-off values for prognostic variables or cut-offs should be avoided.*

We have analysed individual immunoscores as continuous variables with no cut-off. The use of cut-off for loss of I-MMR was based entirely on this being the middle of the range of scores, but can be criticised as a cut-off.

10. *Confidence intervals should be provided to indicate the uncertainty in estimates.*

The 95% confidence intervals for the hazard ratios are stated for the multivariate analyses.

11. *Claims of subset-specific treatment effects should be documented by a test of the single global null hypothesis that there is no treatment specificity involving any of the subsetting variables.*

We have not used any subsets of patients in our analyses.

Criticisms of the immunoscoring technique.

We have used a semi-quantitative method to look at both intensity and percentage of cells showing positive immunostain. We found the score for intensity of staining of MLH1 to be of more prognostic value than the score for percentage of cells stained. This may have been due to the limited number of categories in the percentage scoring system used. The correlation of the immunostain score with MSI or protein expression by Western analysis has not been studied in this group, but in a previous study in 297 colorectal tumours there was a 100% specificity and 100% sensitivity of immunostaining to predict MSI (Cawkwell, Gray et al. 1999). Summarising all the available studies we found immunohistochemical analysis to have a 98.9% power to predict MSI. The studies for immunohistochemistry to predict MMR gene mutation are much more limited but we found a positive predictive power of 76%. It is not known what level of expression of MMR proteins is required to affect immunostaining, to produce MSI or to affect drug sensitivity. Interestingly it has been shown in a melanoma cell line that resistance to cisplatin, etoposide and vindesine is associated with reduction in MMR proteins; MLH1, MSH2 and MSH6 as shown by Western blot analysis, but does not give rise to the MSI phenotype (Lage, Christmann et al. 1999). In this model a reduction in MMR rather than complete loss was associated with chemotherapy resistance. More work on the relevance of immunostaining to predict functionally important loss of MMR is required.

3. RELATION TO OTHER STUDIES

In vitro it has been shown that ovarian tumour cell lines, which have acquired cisplatin resistance, also frequently show loss of MLH1 compared to parental cell lines (Aebi, Kurdi-Haidar et al. 1996; Brown, Hirst et al. 1997). A recent study of 54 ovarian cancer patients treated with platinum based chemotherapy, did not show an association of prechemotherapy MLH1 or MSH2 immunohistochemical staining with overall survival (Samimi, Fink et al. 2000). There are differences between this previous study and the present one in terms of the patient populations, chemotherapy regimens and immunohistochemistry scoring, and there are also differences in how the data have been analysed. In the Samimi study all patients were FIGO stage 3 or 4 whereas 26% of the patients in our study were stage IC or II. Their patients were selected from studies involving second look laparotomy after at least 2 cycles of chemotherapy, which suggests, (although not stated), that the majority of patients had residual disease after their initial laparotomy. By contrast one third of our patients had minimal residual disease. Both of the above suggest that their population was of a more advanced, and therefore poorer prognostic stage. Their patient population received either cisplatin or carboplatin (doses not stated) based chemotherapy as first line treatment. It is unknown if any of the patients also received taxoids. Our results were stratified for cisplatin regimen, to avoid any bias from dose of chemotherapy, which has been shown to be important for survival (Kaye, Paul et al. 1996). For immunohistochemical scoring they used a single scorer, blinded to clinical outcome. They used an intensity scale from 0-4 compared to our study of 0-3. For percentage of cells they used decades (0-100% in 10% increments) compared to our 0-3 scale. This more extensive scale may have lent itself to more accurate interpretation but

unfortunately no assessment of variability of scoring such as a kappa scoring was included, and inter-observer variability was not examined.

On analysis they confirm our finding that intensity and percentage staining for MMR proteins are closely correlated. This suggests the two staining characteristics are not independent of each other. They also confirm that hMLH1 staining is positively correlated with hMSH2 staining, suggesting there is coordinated regulation of MMR protein expression in ovarian cancer.

Of contrast, in particular, the present study has identified a significant association with survival using multivariate analysis of the combined MLH1 and MSH2 immunoscores. Loss of either MLH1 or MSH2 can lead to loss of MMR. We have also shown that loss of MLH1 is associated with poor overall survival only on multivariate analysis. The previous study used only univariate analysis of the individual proteins (Samimi, Fink et al. 2000). Multivariate analysis is necessary to uncover any significant associations that may be masked by other clinical features that affect response to chemotherapy. Thus, we have shown a correlation of a low score for MLH1 with early stage (i.e. IC or II), which is, by itself, a good prognostic factor. The correlation of MMR deficiency and early stage was noted in a previous study of microsatellite instability in ovarian neoplasms (King, Carcangiu et al. 1995) with 75% of stage I patients showing MSI compared to 11% of stage II, III or IV tumours ($p=0.01$). Because of this association of loss of MMR with stage, if only a univariate analysis of MMR expression was considered any correlation of MMR with survival may be obscured. Indeed on univariate analysis we do not observe an association with survival. It is only on correcting for clinical prognostic factors by multivariate

analysis that the true poor prognostic association with loss of MMR expression is revealed.

Although the patients in the Samimi study were all of an advanced stage, they have not analysed survival corrected for histology, grade of tumour, residual disease, performance status or chemotherapy regimen, all of which may influence survival. They did not show any association of MMR protein staining with FIGO grade or age. They did show an association of lower intensity of MSH2 staining pretreatment in stage 4 compared to stage 3 patients ($p=0.0236$). We did not examine the difference between stage 3 and 4 patients but, by contrast, found a loss of MMR staining with stage IC or II compared to stage 3 or 4. They confirmed our finding that patients with serous adenocarcinoma tended to have a higher expression of MMR proteins. However, they did not include the 'not otherwise specified' subtype histology in their analysis, which comprised 33% of their patients. We found that adenocarcinoma (poorly differentiated) showed a lower expression of MMR proteins. In concordance with our study they found no relationship between pretreatment MLH1 or MSH2 staining and response to chemotherapy.

An increase in the proportion of ovarian tumours negative for MLH1 in samples from patients taken after platinum-based chemotherapy compared to untreated tumours has been previously shown (Brown, Hirst et al. 1997; Fink, Nebel et al. 1998; Samimi, Fink et al. 2000). In our study we saw no significant change in pre and post chemotherapy staining for MLH1 or MSH2. However, in a similar clinical study Samimi et al. (Samimi, Fink et al. 2000) showed a mean reduction of 15% for MSH2 and 18.7% for MLH1. It is clearly possible that we lost the sensitivity to detect such a change by our 0-3 scale for percentage staining. In particular, any changes between

20 and 80% would have all fallen into category 2. Interestingly, their study showed no difference in change of MMR proteins regardless of whether the biopsy was within 4 months or 10 months after chemotherapy. They showed no correlation of change in MMR staining with chemotherapy and survival in keeping with our study. In contrast, loss of MLH1 staining, shown in post chemotherapy samples from breast cancer patients receiving neoadjuvant chemotherapy, was a predictor for poor disease free survival (Mackay, Cameron et al. 2000). Counterintuitively, in the Samimi study loss of intensity or percentage of MLH1 staining in the post chemotherapy samples was found to be associated with a good response to treatment ($p=0.0081$ and 0.0178 respectively). However, this result was not subject to a multivariate analysis for other predictors of chemotherapy response. In our study we found a similar contrary result that a reduction in intensity of MSH2 staining post chemotherapy was associated with a good progression free survival on multivariate analysis. These results cannot be easily explained. It is difficult to know what residual tumour represents at second look laparotomy in ovarian cancer patients, especially as early as after 2 cycles of chemotherapy as in the Samimi study. Some residual tumour may represent resistant disease whereas other tumour may represent responding, but not yet completely responding, tumour.

In ovarian cancer patients a correlation between positive p53 immunohistochemistry or p53 mutations of tumours and poor response to cisplatin/carboplatin based chemotherapy has been shown (Righetti, Torre et al. 1996), although studies on p53 status and overall survival have been conflicting (see table 18). Consistent with some studies, p53 immunostaining showed no significant independent prognostic value in our study (Hartmann, Podratz et al. 1994; Sheridan, Silcocks et al. 1994; van der Zee, Hollema et al. 1995; Goff, Muntz et al. 1998; Marx, Meden et al. 1998) and was

independent of expression of either MLH1 or MSH2 or Ki67. Immunohistochemical detection of p53 does not necessarily indicate expression of a mutated protein, but it may be a useful marker for the presence of a functionally abnormal p53 protein (Wynford-Thomas 1992; Hall and Lane 1994). The difference in these results may be due to the use of different antibodies and antigen retrieval techniques (Lambkin, Mothersill et al. 1994; Baas, van den Berg et al. 1996) or due to difference in scoring of immunostaining (Geisler, Geisler et al. 1997). The only previous study using the same antibody as in the current study (Ab-6) used a cut-off point of over 20% cells staining as positive, whereas we treated both the intensity and percentage scores as continuous variables (Rohlke, Milde-Langosch et al. 1997). They also used overnight incubation as opposed to our microwave antigen retrieval. In 104 ovarian cancer patients they found an association of 'positive' p53 staining and poor overall survival on multivariate analysis ($p=0.014$). In their multivariate analysis they included histological grade but not performance status or residual disease as important clinical prognostic factors. Our study may have been limited due to number of patients.

Following the observation on light microscopy of differential expression of MMR genes in normal tissue with a tendency to increased expression in the proliferating compartments e.g. crypts of Lieberkuhn, we felt it was important to stain the material for Ki67 as a surrogate marker for proliferation (Wilson, Ewel et al. 1995; Leach, Polyak et al. 1996). Our finding of a positive correlation of increasing MLH1 and MSH2 scores with increasing Ki67 scores lend support to this observation. Six studies have shown increased Ki67 expression in ovarian cancer to be associated with poor overall survival on univariate analysis (Jordan, Kerns et al. 1993; Kerns, Jordan et al. 1994; Altavilla, Marchetti et al. 1996; Layfield, Saria et al. 1997; Anttila, Kosma

et al. 1998; Goff, Muntz et al. 1998). However, the association of increased Ki67 expression with advanced stage, (a poor prognostic indicator), as found in the current study, is confirmed by Altavilla et al. (Altavilla, Marchetti et al. 1996). Only one study performed a multivariate analysis including stage (Anttila, Kosma et al. 1998). It showed an independent association of increased Ki67 expression and overall survival but the p value was marginal (p=0.04). Our study did not support Ki67 as an independent prognostic indicator in keeping with a previous study by Rohlke et al.(Rohlke, Milde-Langosch et al. 1997).

4. MEANING OF STUDY AND POSSIBLE MECHANISMS

How mismatch repair deficiency could lead to the development of drug resistance is not yet fully understood. The mismatch repair protein MutSα (a heterodimer of MSH2 and MSH6) recognizes and binds to sites of DNA damage such as O^6meG and 1,2 cisplatin intrastrand crosslinks (Duckett, Drummond et al. 1996; Yamada, O'Regan et al. 1997). This has been suggested to lead to either futile rounds of DNA repair (Karran and Hampson 1996), replication stalling (Brown, Hirst et al. 1997), or reduced replication bypass (Vaisman, Varchenko et al. 1998; Durant, Morris et al. 1999) causing increased activation of an apoptotic pathway. An alternative explanation for the correlation of loss of MMR with drug resistance is that loss of MMR leads to a mutator phenotype and therefore may be a surrogate marker for other changes leading to a more aggressive tumour. However, restoration of MMR by chromosome transfer in deficient cells restores drug sensitivity including to cisplatin, arguing for a direct role for MMR in determining cell death and chemosensitivity (Aebi, Kurdi-Haidar et al. 1996; Fink, Nebel et al. 1996). More recently restoration of the MLH1 gene itself into MLH1 deficient strain of *Saccharomyces cerevisiae* has been shown to increase sensitivity to cisplatin compared to vector alone control (Durant, Morris et al. 1999).

The control of expression of MMR genes is as yet poorly understood. Recent observations have shown that the promoter of the MLH1 gene undergoes hypermethylation in ovarian, colorectal and gastric tumours as well as cisplatin resistant cell lines and that this correlates with loss of MLH1 expression (Kane, Loda et al. 1997; Leung, Yuen et al. 1999; Strathdee, Mackean et al. 1999). It is of note

that cisplatin itself can cause hypermethylation of such CpG rich promoter regions (Nyce 1989). This may explain why MLH1, out of all of the MMR proteins, is frequently lost in chemotherapy resistant models.

5. UNANSWERED QUESTIONS AND FUTURE RESEARCH

Our study demonstrates the potential clinical prognostic value of loss of MLH1 and MSH2, as shown by immunohistochemistry, in pre-chemotherapy samples from ovarian cancer patients. Prospective, independent studies are required to confirm this finding and are ongoing. If the association of loss of MMR and poor survival and resistance is confirmed, future treatment strategies could specifically address the issue of targeting cells with loss of MMR or could involve the development of agents which are active in MMR deficient tumour cells e.g. taxoids, topo I – isomerase inhibitors or oxaliplatin.

A larger study is needed to examine the clinical significance of reduction in MLH1 after chemotherapy. Particular care should be taken to examining why the second laparotomy has been performed. The use of decades instead of a 4 point scale for percentage of cells positive for MMR proteins should be explored, including inter-observer and intra-observer variation.

Work with serum DNA in ovarian cancer patients is ongoing. It remains to be established whether this does indeed represent tumour DNA. The clinical usefulness of this, e.g. to predict overall survival or response to chemotherapy, then needs to be explored.

Chapter 10 – REFERENCES

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APPENDIX I – PUBLICATIONS RELATED TO THIS THESIS

Combined MSH2/MLH1 expression in ovarian tumours treated with primary cisplatin chemotherapy predicts disease-free and overall survival.

MJ Mackean, DWM Millan, J Paul, JA Davis, SB Kaye and R Brown

Submitted to Journal of Clinical Investigation

Methylation of the hMLH1 promoter results in loss of hMLH1 expression and drug resistance in ovarian tumour cells.

G Strathdee, **MJ Mackean**, M Illand and R Brown

Oncogene 18: 2335-2341, 1999

Reduced MLH1 expression in breast tumours after primary chemotherapy predicts disease free survival.

HJ Mackay, D Cameron, M Rahilly, **MJ Mackean**, J Paul, SB Kaye and R Brown.

J Clin Oncol 18:87-93, 2000

Immunostaining human paraffin embedded sections for mismatch repair proteins.

MJ Mackean, R Brown.

Invited chapter in Methods in Molecular Biology, In press.

Editor: Prof. JM Walker

Abstracts presented

Methylation of the MLH1 promoter results in loss of MLH1 expression and drug resistance in ovarian tumour cells.

G Strathdee, **MJ Mackean** and R Brown

British Association for Cancer Research meeting. June 1998, Dublin

BJ Cancer 78 (suppl 1), #7.1, p20, 1998

Mismatch repair protein immunohistochemistry in ovarian cancer.

MJ Mackean, D Millan, SB Kaye and R Brown.

British Association for Cancer Research meeting. June 1998, Dublin

BJ Cancer 78 (suppl 1), #P11, p27, 1998

The clinical relevance of mismatch repair protein immunohistochemistry in ovarian cancer.

MJ Mackean, D Millan, J Paul, SB Kaye and R Brown

American Association of Cancer Research. March 1999, Philadelphia

Proc. AACR 40, #3285, p498, 1999

Reduced MLH1 expression in breast tumors after primary chemotherapy predicts disease free survival.

HJ Mackay, D Cameron, M Rawhilly, **MJ Mackean**, J Paul, SB Kaye and R Brown

American Association of Cancer Research. March 1999, Philadelphia

Proc. AACR 40, #3251, p492, 1999

The role of methylation of the hMLH1 promoter in loss of hMLH1 expression and drug resistance in ovarian cancer.

G Strathdee, **MJ Mackean**, M Illand and R Brown

American Association of Cancer Research. March 1999, Philadelphia

Proc. AACR 40, #4435, p672, 1999